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Applicant:
(Name and address)

Novo Nordisk A/S
Novo Allé
DK-2880 Bagsværd
Denmark

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Susanne Morsing

PATENT- OG VAREMÆRKESTYRELSEN

Novel GLP-1 derivatives

FIELD OF THE INVENTION

The present invention relates to novel derivatives of glucagon-like-peptide-1 (GLP-1) and fragments thereof and analogues of such fragments which have a protracted profile of action and methods of making and using them. The invention furthermore relates to novel derivatives of exendin and the use of such derivatives.

BACKGROUND OF THE INVENTION

Peptides are widely used in medical practice, and since they can be produced by recombinant DNA technology it can be expected that their importance will increase also in the years to come. When native peptides or analogues thereof are used in therapy it is generally found that they have a high clearance. A high clearance of a therapeutic agent is inconvenient in cases where it is desired to maintain a high blood level thereof over a prolonged period of time since repeated administrations will then be necessary. Examples of peptides which have a high clearance are: ACTH, corticotropin-releasing factor, angiotensin, calcitonin, insulin, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, insulin-like growth factor-1, insulin-like growth factor-2, gastric inhibitory peptide, growth hormone-releasing factor, pituitary adenylate cyclase activating peptide, secretin, enterogastrin, somatostatin, somatotropin, somatomedin, parathyroid hormone, thrombopoietin, erythropoietin, hypothalamic releasing factors, prolactin, thyroid stimulating hormones, endorphins, enkephalins, vasopressin, oxytocin, opiods and analogues thereof, superoxide dismutase, interferon, asparaginase, arginase, arginine deaminase, adenosine deaminase and ribonuclease. In some cases it is possible to influence the release profile of peptides by applying suitable pharmaceutical compositions, but this approach has various shortcomings and is not generally applicable.

The hormones regulating insulin secretion belong to the so-called enteroinsular axis, designating a group of hormones, released from the gastrointestinal mucosa in response to the presence and absorption of nutrients in the gut, which promote an early and potentiated release of insulin. The enhancing effect on insulin secretion, the so-called incretin effect, is probably essential for a normal glucose tolerance. Many of the gastrointestinal hormones, including gastrin and secretin (cholecystokinin is not insulinotropic in man), are insulinotropic, but the only physiologically important ones, those that are responsible for the incretin effect, are the glucose-dependent insulinotropic polypeptide, GIP, and glucagon-like peptide-

1 (GLP-1). Because of its insulinotropic effect, GIP, isolated in 1973 (1) immediately attracted considerable interest among diabetologists. However, numerous investigations carried out during the following years clearly indicated that a defective secretion of GIP was not involved in the pathogenesis of insulin dependent diabetes mellitus (IDDM) or non insulin-dependent diabetes mellitus (NIDDM) (2). Furthermore, as an insulinotropic hormone, GIP was found to be almost ineffective in NIDDM (2). The other incretin hormone, GLP-1 is the most potent insulinotropic substance known (3). Unlike GIP, it is surprisingly effective in stimulating insulin secretion in NIDDM patients. In addition, and in contrast to the other insulinotropic hormones (perhaps with the exception of secretin) it also potently inhibits glucagon secretion. Because of these actions it has pronounced blood glucose lowering effects particularly in patients with NIDDM.

GLP-1, a product of the proglucagon (4), is one of the youngest members of the secretin-VIP family of peptides, but is already established as an important gut hormone with regulatory function in glucose metabolism and gastrointestinal secretion and metabolism (5). The glucagon gene is processed differently in the pancreas and in the intestine. In the pancreas (9), the processing leads to the formation and parallel secretion of 1) glucagon itself, occupying positions 33-61 of proglucagon (PG); 2) an N-terminal peptide of 30 amino acids (PG (1-30)) often called glicentin-related pancreatic peptide, GRPP (10, 11); 3) a hexapeptide corresponding to PG (64-69); 4) and, finally, the so-called major proglucagon fragment (PG (72-158)), in which the two glucagon-like sequences are buried (9). Glucagon seems to be the only biologically active product. In contrast, in the intestinal mucosa, it is glucagon that is buried in a larger molecule, while the two glucagon-like peptides are formed separately (8). The following products are formed and secreted in parallel: 1) glicentin, corresponding to PG (1-69), with the glucagon sequence occupying residues Nos. 33-61 (12); 2) GLP-1(7-36)amide (PG (78-107))amide (13), not as originally believed PG (72-107)amide or 108, which is inactive). Small amounts of C-terminally glycine-extended but equally bioactive GLP-1(7-37), (PG (78-108)) are also formed (14); 3) intervening peptide-2 (PG (111-122)amide) (15); and 4) GLP-2 (PG (126-158)) (15, 16). A fraction of glicentin is cleaved further into GRPP (PG (1-30)) and oxyntomodulin (PG (33-69)) (17, 18). Of these peptides, GLP-1, has the most conspicuous biological activities.

Being secreted in parallel with glicentin/enteroglucagon, it follows that the many studies of enteroglucagon secretion (6, 7) to some extent also apply to GLP-1 secretion, but GLP-1 is metabolised more quickly with a plasma half-life in humans of 2 min (19). Carbohydrate or fat-rich meals stimulate secretion (20), presumably as a result of direct interaction of yet unabsorbed nutrients with the microvilli of the open-type L-cells of the gut mucosa. Endocrine

or neural mechanisms promoting GLP-1 secretion may exist but have not yet been demonstrated in humans.

The incretin function of GLP-1(29-31) has been clearly illustrated in experiments with the GLP-1 receptor antagonist, exendin 9-39, which dramatically reduces the incretin effect elicited by oral glucose in rats (21, 22). The hormone interacts directly with the β-cells via the GLP-1 receptor (23) which belongs to the glucagon/VIP/calcitonin family of G-protein-coupled 7-transmembrane spanning receptors. The importance of the GLP-1 receptor in regulating insulin secretion was illustrated in recent experiments in which a targeted disruption of the GLP-1 receptor gene was carried out in mice. Animals homozygous for the disruption had greatly deteriorated glucose tolerance and fasting hyperglycaemia, and even heterozygous animals were glucose intolerant (24). The signal transduction mechanism (25) primarily involves activation of adenylate cyclase, but elevations of intracellular Ca^{2+} are also essential (25, 26). The action of the hormone is best described as a potentiation of glucose stimulated insulin release (25), but the mechanism that couples glucose and GLP-1 stimulation is not known. It may involve a calcium-induced calcium release (26, 27). As already mentioned, the insulinotropic action of GLP-1 is preserved in diabetic β-cells. The relation of the latter to its ability to convey "glucose competence" to isolated insulin-secreting cells (26, 28), which respond poorly to glucose or GLP-1 alone, but fully to a combination of the two, is also not known. Equally importantly, however, the hormone also potently inhibits glucagon secretion (29). The mechanism is not known, but seems to be paracrine, via neighbouring insulin or somatostatin cells (25). Also the glucagonostatic action is glucose-dependent, so that the inhibitory effect decreases as blood glucose decreases. Because of this dual effect, if the plasma GLP-1 concentrations increase either by increased secretion or by exogenous infusion the molar ratio of insulin to glucagon in the blood that reaches the liver via the portal circulation is greatly increased, whereby hepatic glucose production decreases (30). As a result blood glucose concentrations decrease. Because of the glucose dependency of the insulinotropic and glucagonostatic actions, the glucose lowering effect is self-limiting, and the hormone, therefore, does not cause hypoglycaemia regardless of dose (31). The effects are preserved in patients with diabetes mellitus (32), in whom infusions of slightly supraphysiological doses of GLP-1 may completely normalise blood glucose values in spite of poor metabolic control and secondary failure to sulphonylurea (33). The importance of the glucagonostatic effect is illustrated by the finding that GLP-1 also lowers blood glucose in type-1 diabetic patients without residual β-cell secretory capacity (34).

In addition to its effects on the pancreatic islets, GLP-1 has powerful actions on the gastrointestinal tract. Infused in physiological amounts, GLP-1 potently inhibits pentagastrin-induced as well as meal-induced gastric acid secretion (35, 36). It also inhibits gastric emptying rate

and pancreatic enzyme secretion (36). Similar inhibitory effects on gastric and pancreatic secretion and motility may be elicited in humans upon perfusion of the ileum with carbohydrate- or lipid-containing solutions (37, 38). Concomitantly, GLP-1 secretion is greatly stimulated, and it has been speculated that GLP-1 may be at least partly responsible for this so-called "ileal-brake" effect (38). In fact, recent studies suggest that, physiologically, the ileal-brake effects of GLP-1 may be more important than its effects on the pancreatic islets. Thus, in dose response studies GLP-1 influences gastric emptying rate at infusion rates at least as low as those required to influence islet secretion (39).

GLP-1 seems to have an effect on food intake. Intraventricular administration of GLP-1 profoundly inhibits food intake in rats (40, 42). This effect seems to be highly specific. Thus, N-terminally extended GLP-1 (PG 72-107)amide is inactive and appropriate doses of the GLP-1 antagonist, exendin 9-39, abolish the effects of GLP-1 (41). Acute, peripheral administration of GLP-1 does not inhibit food intake acutely in rats (41, 42). However, it remains possible that GLP-1 secreted from the intestinal L-cells may also act as a satiety signal.

Not only the insulinotropic effects but also the effects of GLP-1 on the gastrointestinal tract are preserved in diabetic patients (43), and may help curtailing meal-induced glucose excursions, but, more importantly, may also influence food intake. Administered intravenously, continuously for one week, GLP-1 at 4 ng/kg/min has been demonstrated to dramatically improve glycaemic control in NIDDM patients without significant side effects (44). The peptide is fully active after subcutaneous administration (45), but is rapidly degraded mainly due to degradation by dipeptidyl peptidase IV-like enzymes (46, 47).

The amino acid sequence of GLP-1 is given *i.a.* by Schmidt *et al.* (*Diabetologia* **28** 704-707 (1985). Although the interesting pharmacological properties of GLP-1(7-37) and analogues thereof have attracted much attention in recent years only little is known about the structure of these molecules. The secondary structure of GLP-1 in micelles has been described by Thorton *et al.* (*Biochemistry* **33** 3532-3539 (1994)), but in normal solution,GLP-1 is considered a very flexible molecule. Surprisingly, we found that derivatisation of this relatively small and very flexible molecule resulted in compounds whose plasma profile were highly protracted and still had retained activity.

GLP-1 and analogues of GLP-1 and fragments thereof are potentially useful *i.a.* in the treatment of type 1 and type 2 diabetes. However, the high clearance limits the usefulness of these compounds, and thus there still is a need for improvements in this field. Accordingly, it is one object of the present invention to provide derivatives of GLP-1 and analogues thereof which have a protracted profile of action relative to GLP-1(7-37). It is a further object of the invention

to provide derivatives of GLP-1 and analogues thereof which have a lower clearance than GLP-1(7-37). It is a further object of the invention to provide a pharmaceutical composition comprising a compound according to the invention and to use a compound of the invention to provide such a composition. Also, it is an object of the present invention to provide a method of treating insulin dependent and non-insulin dependent diabetes mellitus.

The number of known endogenous peptides and proteins with interesting biological activities is growing rapidly, also as a result of the ongoing exploration of the human genome. Due to their biological activities, many of these peptides and proteins could in principle be used as therapeutic agents. Endogenous peptides are, however, not always suitable as drug candidates because these peptides often have halflives of few minutes due to rapid degradation by peptidases and/or due to renal filtration and excretion in the urine. It has been shown (Zobel et al., *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1513-1515) that the plasma halflife of a given peptide may be significantly enhanced by reversible attachment of this peptide to plasma proteins, such as albumin or gamma-globulin. Human serum albumin (HSA) has, for instance, a halflife of more than one week. This reversible attachment requires a compound (= albumin binding residue) which can be linked to the therapeutic agent, and which has a high binding affinity to albumin while bound to said therapeutic agent. Using the known strategies for reversible binding of endogenous proteins such as GLP-1 to albumin it is however often observed that the albumin binding fraction of the therapeutic agent has diminished or no activity on the GLP-1 receptor. One aspect of this invention is to introduce a hydrophilic linker between the therapeutic protein and the albumin binding residue in such a manner that the free fraction as well as the albumin binding fraction of the therapeutic protein are both active on the target which may be a GCPR receptor such as the GLP-1 receptor or an enzyme.

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SUMMARY OF THE INVENTION

The present invention relates to a compound which comprises a therapeutic polypeptide linked to an albumin binding residue via a hydrophilic spacer.

The present invention also relates to a compound which comprises a therapeutic polypeptide linked to an albumin binding residue via a hydrophilic spacer that separates the

polypeptide and the albumin binding residue with a chemical molety comprising at least 5 heavy atoms where 30-50% of these are either N or O.

In one embodiment of this invention the spacer is defined as

$-(CH_2)_lD[(CH_2)_nE]_m(CH_2)_pQ_q-$, wherein

l, m and n independently are 1-20 and p is 0-10,

Q is $-Z-(CH_2)_lD[(CH_2)_nG]_m(CH_2)_p-$,

q is an integer in the range from 0 to 5,

each D, E, and G independently are selected from -O-, $-NR^3-$, $-N(COR^4)-$, $-PR^5(O)-$, and $-P(OR^6)(O)-$, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from $-C(O)NH-$, $-C(O)NHCH_2-$, $-OC(O)NH-$, $-C(O)NHCH_2CH_2-$, $-C(O)CH_2-$, $-C(O)CH=CH-$, $-(CH_2)_s-$, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1

or a pharmaceutically acceptable salt or prodrug thereof.

The present invention also relates to a compound which has the formula (I) :

A—W—B—Y—therapeutic polypeptide (I)

wherein

A is an albumin binding residue,

B is a hydrophilic spacer being $-(CH_2)_lD[(CH_2)_nE]_m(CH_2)_pQ_q-$, wherein

l, m and n independently are 1-20 and p is 0-10,

Q is $-Z-(CH_2)_lD[(CH_2)_nG]_m(CH_2)_p-$,

q is an integer in the range from 0 to 5,

each D, E, and G independently are selected from -O-, $-NR^3-$, $-N(COR^4)-$, $-PR^5(O)-$, and $-P(OR^6)(O)-$, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from $-C(O)NH-$, $-C(O)NHCH_2-$, $-OC(O)NH-$, $-C(O)NHCH_2CH_2-$, $-C(O)CH_2-$, $-C(O)CH=CH-$, $-(CH_2)_s-$, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1,

Y is a chemical group linking B and the therapeutic agent, and

W is a chemical group linking A and B.

The present invention also relates to a compound which has the formula (II)

A—W—B—Y—therapeutic polypeptide —Y′—B′-W′—A′ (II)

wherein

A and A´ are albumin binding residues,

B and B´ are hydrophilic spacers independently selected from $-(CH_2)_lD\,[(CH_2)_nE]_m(CH_2)_p-Q_q-$, wherein

l, m and n independently are 1-20 and p is 0-10,

Q is $-Z-(CH_2)_lD[(CH_2)_nG]_m(CH_2)_p-$,

q is an integer in the range from 0 to 5,

each D, E, and G independently are selected from -O-, $-NR^3-$, $-N(COR^4)-$, $-PR^5(O)-$, and $-P(OR^6)(O)-$, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from $-C(O)NH-$, $-C(O)NHCH_2-$, $-OC(O)NH-$, $-C(O)NHCH_2CH_2-$, $-C(O)CH_2-$, $-C(O)CH=CH-$, $-(CH_2)_s-$, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1,

Y is a chemical group linking B and the therapeutic agent, and

Y´ is a chemical group linking B´ and the therapeutic agent, and

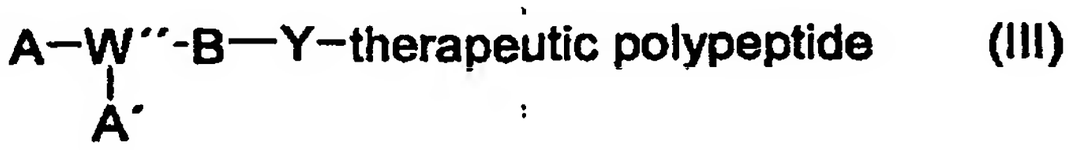
W is a chemical group linking A and B, and

W´ is a chemical group linking A´ and B´.

In one embodiment of the invention Y´ is selected from the group consisting of $-C(O)NH-$, $-NHC(O)-$, $-C(O)NHCH_2-$, $-CH_2NHC(O)-$, $-OC(O)NH-$, $-NHC(O)O-$, $-C(O)NHCH_2-$, $CH_2NHC(O)-$, $-C(O)CH_2-$, $-CH_2C(O)-$, $-C(O)CH=CH-$, $-CH=CHC(O)-$, $-(CH_2)_s-$, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and $-C(O)NH-$, wherein s is 0 or 1.

In a further embodiment of the invention W´ is selected from the group consisting of $-C(O)NH-$, $-NHC(O)-$, $-C(O)NHCH_2-$, $-CH_2NHC(O)-$, $-OC(O)NH-$, $-NHC(O)O-$, $-C(O)CH_2-$, $CH_2C(O)-$, $-C(O)CH=CH-$, $-CH=CHC(O)-$, $-(CH_2)_s-$, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and $-C(O)NH-$, wherein s is 0 or 1.

In another aspect the present invention relates to a compound which has the formula (III)



wherein

A and A´ are albumin binding residues,

B is a hydrophilic spacer selected from $-(CH_2)_lD[(CH_2)_nE]_m(CH_2)_p-Q_q-$ wherein

l, m and n independently are 1-20 and p is 0-10,

Q is $-Z-(CH_2)_lD[(CH_2)_nG]_m(CH_2)_p-$,

q is an integer in the range from 0 to 5,

each D, E, and G are independently selected from -O-, -NR^3-, -$N(COR^4)$-, -$PR^5(O)$-, and -$P(OR^6)(O)$-, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from -C(O)NH-, -$C(O)NHCH_2$-, -OC(O)NH -, -$C(O)NHCH_2CH_2$-, -$C(O)CH_2$-, -C(O)CH=CH-, -$(CH_2)_6$-, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1,

Y is a chemical group linking B and the therapeutic agent, and

W'' is a chemical group linking B with A' and A'.

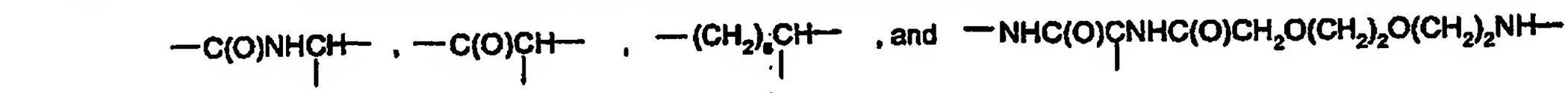
In another aspect the present invention relates to a compound comprising a hydrophilic spacer between a therapeutic peptide and one or more albumin binding residue(s), said compound having a protracted profile of action relative to the therapeutic polypeptide, where the albumin binding fraction as well as the free fraction of said compound are both able to bind to the receptor mediating the effect of the therapeutic polypeptide.

In one embodiment the hydrophilic spacer is an unbranched oligo ethylene glycol moiety with appropiate funtional groups at both terminals that forms a bridge between an amino group of the therapeutic polypeptide and a funtional group of the albumin binding residue.

In one embodiment Y is selected from the group consisting of -C(O)NH-, -NHC(O)-, -$C(O)NHCH_2$-, -$CH_2NHC(O)$-, -OC(O)NH -, -NHC(O)O-, -$C(O)NHCH_2$-, $CH_2NHC(O)$-, -$C(O)CH_2$-, -$CH_2C(O)$-, -C(O)CH=CH-, -CH=CHC(O)-, -$(CH_2)_s$-, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and –C(O)NH-, wherein s is 0 or 1.

In another embodiment W'' is selected from the group consisting of -NHC(O)O-, -$C(O)CH_2$-, -$CH_2C(O)$-, -C(O)CH=CH-, -CH=CHC(O)-, -$(CH_2)_s$-, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and –C(O)NH-, wherein s is 0 or 1.

In another embodiment W'' is selected from the group consisting of



, wherein s is 0, 1 or 2.

In another embodiment l is 1 or 2, n and m are independently 1-10 and p is 0-10.

In another embodiment D is –O-.

In another embodiment of the invention E is –O-.

In yet another embodiment of the invention the hydrophilic spacer is $-CH_2O[(CH_2)_2O]_m(CH_2)_pQ_q-$, where m is 1-10, p is 1-3, and Q is $-Z-CH_2O[(CH_2)_2O]_m(CH_2)_p-$.

In another embodiment q is 1.

In another embodiment G is –O-.

In yet another embodiment of the invention Z is selected from the group consisting of -C(O)NH-, $-C(O)NHCH_2-$, and –OC(O)NH-.

In yet another embodiment q is 0.

In another embodiment l is 2.

In another embodiment n is 2.

In yet another embodiment the hydrophilic spacer B is $-[CH_2CH_2O]_{m+1}(CH_2)_pQ_q-$.

In another embodiment the molar weight of said hydrophilic spacer is in the range from 80D to 1000D or in the range from 80D to 300D.

In another aspect of the present invention the therapeutic polypeptide is a GLP-1 peptide.

DEFINITIONS

In the present specification, the following terms have the indicated meaning :

The term "albumin binding residue" as used herein means a residue which binds non-covalently to human serum albumin. The albumin binding residue attached to the therapeutic polypeptide typically has an affinity below 10 µM to human serum albumin and preferably below 1 µM. A range of albumin binding residues are known among linear and branched lipophillic moieties containing 4-40 carbon atoms, compounds with a cyclopentanophenanthrene skeleton, peptides having 10-30 amino acid residues etc.

The term "hydrophilic spacer" as used herein means a spacer that separates a peptide and an albumin binding residue with a chemical moiety which comprises at least 5 heavy atoms where 30-50% of these are either N or O.

The term "therapeutic polypeptide" as used herein means a polypeptide which is being developed for therapeutic use, or which has been developed for therapeutic use.

The term "polypeptide" and "peptide" as used herein means a compound composed of at least five constituent amino acids connected by peptide bonds. The constituent amino acids may be from the group of the amino acids encoded by the genetic code and they may natural amino acids which are not encoded by the genetic code, as well as synthetic amino acids. Natural amino acids which are not encoded by the genetic code are e.g. hydroxyproline, *γ*-carboxyglutamate, ornithine, phosphoserine, D-alanine and D-glutamine. Synthetic amino ac-

ids comprise amino acids manufactured by chemical synthesis, i.e. D-isomers of the amino acids encoded by the genetic code such as D-alanine and D-leucine, Aib (α-aminoisobutyric acid), Abu (α-aminobutyric acid), Tle (tert-butylglycine), β-alanine, 3-aminomethyl benzoic acid, anthranilic acid.

The term "analogue" as used herein referring to a polypeptide means a modified peptide wherein one or more amino acid residues of the peptide have been substituted by other amino acid residues and/or wherein one or more amino acid residues have been deleted from the peptide and/or wherein one or more amino acid residues have been deleted from the peptide and or wherein one or more amino acid residues have been added to the peptide. Such addition or deletion of amino acid residues can take place at the N-terminal of the peptide and/or at the C-terminal of the peptide. Two different and simple systems are often used to describe analogues : For example Arg^{34}-GLP-1(7-37) or K34R-GLP-1(7-37) designates a GLP-1 analogue wherein amino acid residues at position 1-6 have been deleted, and the naturally occuring lysine at position 34 has been substituted with arginine (standard single letter abbreviation for amino acids used according to IUPAC-IUB nomenclature).

The term "derivative" as used herein in relation to a peptide means a chemically modified peptide or an analogue thereof, wherein at least one substituent is not present in the unmodified peptide or an analogue thereof, i.e. a peptide which has been covalently modified. Typical modifications are amides, carbohydrates, alkyl groups, acyl groups, esters and the like. An example of a derivative of GLP-1(7-37) is Arg^{34}, $Lys^{26}(N^{\varepsilon}$-(γ-Glu(N^{α}-hexadecanoyl)))-GLP-1(7-37).

The term "GLP-1 peptide" as used herein means GLP-1(7-37), a GLP-1 analogue, a GLP-1 derivative or a derivative of a GLP-1 analogue. In one embodiment the GLP-1 peptide is an insulinotropic agent.

The term "insulinotropic agent" as used herein means a compound which is an agonist of the human GLP-1 receptor, i.e. a compound which stimulates the formation of cAMP in a suitable medium containing the human GLP-1 receptor. The potency of an insulinotropic agent is determined by calculating the EC_{50} value from the dose-response curve as described below.

Baby hamster kidney (BHK) cells expressing the cloned human GLP-1 receptor (BHK-467-12A) were grown in DMEM media with the addition of 100 IU/mL penicillin, 100 µL/mL streptomycin, 10% fetal calf serum and 1 mg/mL Geneticin G-418 (Life Technologies). Plasma membranes were prepared by homogenisation in buffer (10 mM Tris-HCl, 30 mM NaCl and 1 mM dithiothreitol, pH 7.4, containing, in addition, 5 mg/L leupeptin (Sigma, St. Louis, MO, USA), 5 mg/L pepstatin (Sigma), 100 mg/L bacitracin (Sigma), and 16 mg/L aprotinin (Calbiochem-Novabiochem, La Jolla, CA). The homogenate was centrifuged on top of a layer of 41 w/v%

sucrose. The white band between the two layers was diluted in buffer and centrifuged. Plasma membranes were stored at -80°C until used.

The functional receptor assay was carried out by measuring cAMP as a response to stimulation by the insulinotropic agent. Incubation were carried out in 96-well microtiter plates in a total volume of 140 µL and with the following final concentrations: 50 mM Tris-HCl, 1 mM EGTA, 1.5 mM $MgSO_4$, 1.7 mM ATP, 20 mM GTP, 2 mM 3-isobutyl-1-methylxanthine (IBMX), 0.01 % Tween-20, pH 7.4. Compounds to be tested for agonist activity were dissolved and diluted in buffer. GTP was freshly prepared for each experiment : 2.5 µg of membrane was added to each well and the mixture was incubated for 90 min at room temperature in the dark with shaking. The reaction was stopped by the addition of 25 µL of 0.5 M HCl. Formed cAMP was measured by a scintillation proximity assay (RPA 542, Amersham, UK). Dose-response curves were plotted for the individual compounds and EC_{50} values calculated using GraphPad Prism software.

The term "GLP-2 peptide" as used herein means GLP-2(1-33), a GLP-2 analogue, a GLP-2 derivative or a derivative of a GLP-2 analogue.

The term "exendin-4 peptide" as used herein means exendin-4(1-39), an exendin-4 analogue, an exendin-4 derivative or a derivative of an exendin-4 analogue. In one embodiment the exendin-4 peptide is an insulinotropic agent.

The terms "stable exendin-4 peptide" and "stable GLP-1 peptides" as used herein means chemically modified peptides derived from exendin-4(1-39) or GLP-1(7-37), i.e. an analogue or a derivative which exhibits an in vivo plasma elimination half-life of at least 10 hours in man, as determined by the following method. The method for determination of plasma elimination half-life of an exendin-4 peptide or a GLP-1 peptide in man is : The peptide is dissolved in an isotonic buffer, pH 7.4, PBS or any other suitable buffer. The dose is injected peripherally, preferably in the abdominal or upper thigh. Blood samples for determination of active peptide are taken at frequent intervals, and for a sufficient duration to cover the terminal elimination part (e.g. Pre-dose, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24 (day 2), 36 (day 2), 48 (day 3), 60 (day 3), 72 (day 4) and 84 (day 4) hours post dose). Determination of the concentration of active peptide is performed as described in Wilken et al., Diabetologia 43(51):A143, 2000. Derived pharmacokinetic parameteres are calculated from the concentration-time data for each individual subject by use of non-compartmental methods, using the commercially available software WinNonlin Version 2.1 (Pharsight, Cary, NC, USA). The terminal elimination rate constant is estimated by log-linear regression on the terminal log-linear part of the concentration-time curve, and used for calculating the elimination half-life.

The term "DPP-IV protected" as used herein referring to a polypeptide means a polypeptide which has been chemically modified in order to render said compound resistant to the

plasma peptidase dipeptidyl aminopeptidase-4 (DPP-IV). The DPP-IV enzyme in plasma is known to be involved in the degradation of several peptide hormones, e.g. GLP-1, GLP-2, Exendin-4 etc. Thus, a considerable effort is being made to develop analogues and derivatives of the polypeptides susceptible to DPP-IV mediated hydrolysis in order to reduce the rate of degradation by DPP-IV.

Resistance of a peptide to degradation by dipeptidyl aminopeptidase IV is determined by the following degradation assay :

Aliquots of the peptide (5 nmol) are incubated at 37 °C with 1 µL of purified dipeptidyl aminopeptidase IV corresponding to an enzymatic activity of 5 mU for 10-180 minutes in 100 µL of 0.1 M triethylamine-HCl buffer, pH 7.4. Enzymatic reactions are terminated by the addition of 5 µL of 10% trifluoroacetic acid, and the peptide degradation products are separated and quantified using HPLC analysis. One method for performing this analysis is : The mixtures are applied onto a Vydac C18 widepore (30 nm pores, 5 µm particles) 250 x 4.6 mm column and eluted at a flow rate of 1 ml/min with linear stepwise gradients of acetonitrile in 0.1% trifluoroacetic acid (0% acetonitrile for 3 min, 0-24% acetonitrile for 17 min, 24-48% acetonitrile for 1 min) according to Siegel et al., Regul. Pept. 1999;79:93-102 and Mentlein et al. Eur. J. Biochem. 1993;214:829-35. Peptides and their degradation products may be monitored by their absorbance at 220 nm (peptide bonds) or 280 nm (aromatic amino acids), and are quantified by integration of their peak areas related to those of standards. The rate of hydrolysis of a peptide by dipeptidyl aminopeptidase IV is estimated at incubation times which result in less than 10% of the peptide being hydrolysed.

The term "C_{1-6}-alkyl" as used herein means a saturated, branched, straight or cyclic hydrocarbon group having from 1 to 6 carbon atoms. Representative examples include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, butyl, isobutyl, *sec*-butyl, *tert*-butyl, n-pentyl, isopentyl, neopentyl, *tert*-pentyl, n-hexyl, isohexyl, cyclohexane and the like.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment of the invention, the albumin binding residue is a lipophilic residue.

In another embodiment the albumin binding residue is negatively charged at physiological pH. In another embodiment the albumin binding residue comprises a group which can be negatively charged. One preferred group which can be negatively charged is a carboxylic acid group.

In another embodiment of the invention, the albumin binding residue binds non-covalently to albumin. In another embodiment the albumin binding residue has a binding affinity towards human serum albumin that is below about 10 µM or below about 1 µM.

In yet another embodiment of the invention the albumin binding residue is selected from a straight chain alkyl group, a branched alkyl group, a group which has an ω-carboxylic acid group, a partially or completely hydrogenated cyclopentanophenanthrene skeleton.

In another embodiment the albumin binding residue is a cibacronyl residue.

In another embodiment the albumin binding residue has from 6 to 40 carbon atoms, from 8 to 26 carbon atoms or from 8 to 20 carbon atoms.

In another embodiment the albumin binding residue is an acyl group selected from the group comprising $CH_3(CH_2)_rCO$-, wherein r is an integer from 4 to 38, preferably an integer from 4 to 24, more preferred selected from the group comprising $CH_3(CH_2)_6CO$-, $CH_3(CH_2)_8CO$-, $CH_3(CH_2)_{10}CO$-, $CH_3(CH_2)_{12}CO$-, $CH_3(CH_2)_{14}CO$-, $CH_3(CH_2)_{16}CO$-, $CH_3(CH_2)_{18}CO$-, $CH_3(CH_2)_{20}CO$- and $CH_3(CH_2)_{22}CO$-.

In another embodiment the albumin binding residue is an acyl group of a straight-chain or branched alkane α,ω-dicarboxylic acid.

In another embodiment the albumin binding residue is an acyl group selected from the group comprising $HOOC(CH_2)_sCO$-, wherein s is an integer from 4 to 38, preferably an integer from 4 to 24, more preferred selected from the group comprising $HOOC(CH_2)_{14}CO$-, $HOOC(CH_2)_{16}CO$-, $HOOC(CH_2)_{18}CO$-, $HOOC(CH_2)_{20}CO$- and $HOOC(CH_2)_{22}CO$-.

In another embodiment the albumin binding residue is a group of the formula $CH_3(CH_2)_vCO$-NHCH(COOH)$(CH_2)_2CO$-, wherein v is an integer of from 10 to 24.

In another embodiment the albumin binding residue is a group of the formula $CH_3(CH_2)_wCO$-NHCH($(CH_2)_2COOH$)CO-, wherein w is an integer of from 8 to 24.

In another embodiment the albumin binding residue is a group of the formula $COOH(CH_2)_xCO$- wherein x is an integer of from 8 to 24.

In another embodiment the albumin binding residue is a group of the formula -NHCH(COOH)$(CH_2)_4NH$-$CO(CH_2)_yCH_3$, wherein y is an integer of from 8 to 18.

In another embodiment of the invention the albumin binding residue is a peptide, such as a peptide comprising less than 40 amino acid residues. A number of small peptides which are albumin binding residues as well as a method for their identification is found in J. Biol Chem. 277, 38 (2002) 35035-35043.

In another embodiment of the invention the albumin binding residue via spacer and linkers is attached to said therapeutic polypeptide via the ϵ-amino group of a lysine residue.

In another embodiment the albumin binding residue via spacer and linkers is attached to said therapeutic polypeptide via an amino acid residue selected from cysteine, glutamate and aspartate.

In one embodiment of the present invention the therapeutic polypeptide is a GLP-1 peptide.

In another embodiment of the invention the therapeutic polypeptide is a GLP-1 peptide comprising the amino acid sequence of the formula (IV):

Xaa_7-Xaa_8-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Xaa_{16}-Ser-Xaa_{18}-Xaa_{19}-Xaa_{20}-Glu-Xaa_{22}-Xaa_{23}-Ala-Xaa_{25}-Xaa_{26}-Xaa_{27}-Phe-Ile-Xaa_{30}-Trp-Leu-Xaa_{33}-Xaa_{34}-Xaa_{35}-Xaa_{36}-Xaa_{37}-Xaa_{38}-Xaa_{39}-Xaa_{40}-Xaa_{41}-Xaa_{42}-Xaa_{43}-Xaa_{44}-Xaa_{45}-Xaa_{46}

Formula (IV) (SEQ ID No: 2)

wherein

Xaa_7 is L-histidine, D-histidine, desamino-histidine, 2-amino-histidine, β-hydroxy-histidine, homohistidine, N^{α}-acetyl-histidine, α-fluoromethyl-histidine, α-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine or 4-pyridylalanine;

Xaa_8 is Ala, Gly, Val, Leu, Ile, Lys, Aib, (1-aminocyclopropyl) carboxylic acid, (1-aminocyclobutyl) carboxylic acid, (1-aminocyclopentyl) carboxylic acid, (1-aminocyclohexyl) carboxylic acid, (1-aminocycloheptyl) carboxylic acid, or (1-aminocyclooctyl) carboxylic acid;

Xaa_{16} is Val or Leu;

Xaa_{18} is Ser, Lys or Arg;

Xaa_{19} is Tyr or Gln;

Xaa_{20} is Leu or Met;

Xaa_{22} is Gly, Glu or Aib;

Xaa_{23} is Gln, Glu, Lys or Arg;

Xaa_{25} is Ala or Val;

Xaa_{26} is Lys, Glu or Arg;

Xaa_{27} is Glu or Leu;

Xaa_{30} is Ala, Glu or Arg;

Xaa_{33} is Val or Lys;

Xaa_{34} is Lys, Glu, Asn or Arg;

Xaa_{35} is Gly or Aib;

Xaa_{36} is Arg, Gly or Lys;

Xaa_{37} is Gly, Ala, Glu, Pro, Lys, amide or is absent;

Xaa_{38} is Lys, Ser, amide or is absent.

Xaa_{39} is Ser, Lys, amide or is absent;

Xaa_{40} is Gly, amide or is absent;
Xaa_{41} is Ala, amide or is absent;
Xaa_{42} is Pro, amide or is absent;
Xaa_{43} is Pro, amide or is absent;
Xaa_{44} is Pro, amide or is absent;
Xaa_{45} is Ser, amide or is absent;
Xaa_{46} is amide or is absent ;
provided that if Xaa_{38}, Xaa_{39}, Xaa_{40}, Xaa_{41}, Xaa_{42}, Xaa_{43}, Xaa_{44}, Xaa_{45} or Xaa_{46} is absent then each amino acid residue downstream is also absent.

In another embodiment of the invention the polypeptide is a GLP-1 peptide comprising the amino acid sequence of formula (V):

Xaa_7-Xaa_8-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Xaa_{18}-Tyr-Leu-Glu-Xaa_{22}-Xaa_{23}-Ala-Ala-Xaa_{26}-Glu-Phe-Ile-Xaa_{30}-Trp-Leu-Val-Xaa_{34}-Xaa_{35}-Xaa_{36}-Xaa_{37}-Xaa_{38}

Formula (V) (SEQ ID No: 3)

wherein
Xaa_7 is L-histidine, D-histidine, desamino-histidine, 2-amino-histidine, β-hydroxy-histidine, homohistidine, N^{α}-acetyl-histidine, α-fluoromethyl-histidine, α-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine or 4-pyridylalanine;
Xaa_8 is Ala, Gly, Val, Leu, Ile, Lys, Aib, (1-aminocyclopropyl) carboxylic acid, (1-aminocyclobutyl) carboxylic acid, (1-aminocyclopentyl) carboxylic acid, (1-aminocyclohexyl) carboxylic acid, (1-aminocycloheptyl) carboxylic acid, or (1-aminocyclooctyl) carboxylic acid;
Xaa_{18} is Ser, Lys or Arg;
Xaa_{22} is Gly, Glu or Aib;
Xaa_{23} is Gln, Glu, Lys or Arg;
Xaa_{26} is Lys, Glu or Arg;
Xaa_{30} is Ala, Glu or Arg;
Xaa_{34} is Lys, Glu or Arg;
Xaa_{35} is Gly or Aib;
Xaa_{36} is Arg or Lys;
Xaa_{37} is Gly, Ala, Glu or Lys;
Xaa_{38} is Lys, amide or is absent.

In yet another embodiment of the invention the GLP-1 peptide is selected from GLP-1(7-35), GLP-1(7-36), GLP-1(7-36)-amide, GLP-1(7-37), GLP-1(7-38), GLP-1(7-39), GLP-1(7-40), GLP-1(7-41) or an analogue thereof.

In another embodiment the GLP-1 peptide is a fragment of a peptide selected from the group comprising GLP-1(7-35), GLP-1(7-36), GLP-1(7-36)amide, GLP-1(7-37), GLP-1(7-38), GLP-1(7-39), GLP-1(7-40) and GLP-1(7-41) or an analogue thereof.

In another embodiment of the invention the GLP-1 peptide is GLP-1(A-B) wherein A is an integer from 1 to 7 and B is an integer from 38 to 45 or an analogue thereof comprising one albumin binding residue attached via a hydrophilic spacer to the C-terminal amino acid residue and, optionally, a second albumin binding residue attached to one of the other amino acid residues.

In another embodiment the GLP-1 peptide comprises no more than fifteen amino acid residues which have been exchanged, added or deleted as compared to GLP-1(7-37) (SEQ ID No. 1), or no more than ten amino acid residues which have been exchanged, added or deleted as compared to GLP-1(7-37) (SEQ ID No. 1).

In another embodiment the GLP-1 peptide comprises no more than six amino acid residues which have been exchanged, added or deleted as compared to GLP-1(7-37) (SEQ ID No. 1).

In another embodiment the GLP-1 peptide comprises no more than 4 amino acid residues which are not encoded by the genetic code.

In another embodiment the GLP-1 peptide is a DPPIV protected GLP-1 peptide.

In another embodiment the GLP-1 peptide comprises an Aib residue in position 8.

In another embodiment the amino acid residue in position 7 of said GLP-1 peptide is selected from the group consisting of D-histidine, desamino-histidine, 2-amino-histidine, β-hydroxy-histidine, homohistidine, N^{α}-acetyl-histidine , α-fluoromethyl-histidine, α-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine and 4-pyridylalanine.

In another embodiment the GLP-1 peptide is selected from the group consisting of

Arg^{34}GLP-1(7-37),

$Lys^{38}Arg^{26,34}$GLP-1(7-38), $Lys^{38}Arg^{26,34}$GLP-1(7-38)-OH, $Lys^{36}Arg^{26,34}$GLP-1(7-36),

$Aib^{8,22,35}$GLP-1(7-37), $Aib^{8,35}$GLP-1(7-37), $Aib^{8,22}$GLP-1(7-37),

$Aib^{8,22,35}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Arg^{26,34}Lys^{38}$GLP-1(7-38),

$Aib^{8,22}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,22,35}Arg^{26,34}Lys^{38}$GLP-1(7-38),

$Aib^{8,35}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,22,35}Arg^{26}Lys^{38}$GLP-1(7-38),

$Aib^{8,35}Arg^{26}Lys^{38}$GLP-1(7-38), $Aib^{8,22}Arg^{26}Lys^{38}$GLP-1(7-38),

$Aib^{8,22,35}Arg^{34}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Arg^{34}Lys^{38}$GLP-1(7-38), $Aib^{8,22}Arg^{34}Lys^{38}$GLP-1(7-38),

$Aib^{8,22,35}Ala^{37}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Ala^{37}Lys^{38}$GLP-1(7-38), $Aib^{8,22}Ala^{37}Lys^{38}$GLP-1(7-38),

$Aib^{8,22,35}Lys^{37}$GLP-1(7-37), $Aib^{8,35}Lys^{37}$GLP-1(7-37) and $Aib^{8,22}Lys^{37}$GLP-1(7-38).

In another embodiment the GLP-1 peptide is attached to said hydrophilic spacer via the amino acid residue in position 23, 26, 34, 36 or 38 relative to the amino acid sequence SEQ ID No:1.

In another embodiment the GLP-1 peptide is exendin-4 (SEQ ID NO 4).

In another embodiment the GLP-1 peptide is ZP-10, i.e. HGEGT-FTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPSKKKKKK-amide (SEQ ID NO 5).

In another embodiment the GLP-1 peptide is HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGX, wherein X = P or Y, or a fragment or an analogue thereof.

In another embodiment the parent peptide is DLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS, or a fragment or an analogue thereof.

In another embodiment of the invention the compound is Arg^{18}, Leu^{20}, Gln^{34}, Lys^{33} (N^{ε}-(γ-aminobutyroyl(N^{α}-hexadecanoyl))) Exendin-4-(7-45)-amide or Arg^{33}, Leu^{20}, Gln^{34}, Lys^{18} (N^{ε}-(γ-aminobutyroyl(N^{α}-hexadecanoyl))) Exendin-4-(7-45)-amide.

In another embodiment of the invention one albumin binding residue is attached to the C-terminal amino acid residue of the GLP-1 peptide via the hydrophilic spacer.

In another embodiment of the invention a second albumin binding residue is attached to an amino acid residue which is not the C-terminal amino acid residue of the GLP-1 peptide.

In another embodiment, the lipophilic substituent is attached to the GLP-1 peptide by means of a hydrophilic spacer in such a way that a carboxyl group of the spacer forms an amide bond with an amino group of the GLP-1 peptide.

In another embodiment of the invention the compound is selected from the group consisting of

$N^{\varepsilon 37}$-(2-(2-(2-(dodecylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$]GLP-1(7-37) amide,

$N^{\varepsilon 37}$-(2-(2-(2-(17-sulphohexadecanoylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,Lys37]GLP-1(7-37) amide,

$N^{\varepsilon 37}$-{2-[2-(2-(15-carboxyhexadecanoylamino)ethoxy)ethoxy]acetyl}-[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\varepsilon 37}$-(2-(2-(2-(17-carboxyheptadecanoylamino)ethoxy)ethoxy)acetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\varepsilon 37}$-(2-(2-(2-(19-carboxynonadecanoylamino)ethoxy)ethoxy)acetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\varepsilon 38}$-(4-(hexadecanoylamino)-4(S)-carboxybutyryl)-[$Aib^{8,22,35}$,$Arg^{26,34}$,Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$-(2-(2-(2-(hexadecanoylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,$Arg^{26,34}$,Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 37}$(2-[2-(2,6-(S)-Bis-{2-[2-(2-(dodecanoylamino)ethoxy)ethoxy]acetylamino}hexanoylamino)ethoxy]ethoxy}) acetyl-[$Aib^{8,22,35}$]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(2-[2-(2,6-(S)-Bis-{2-[2-(2-(tetradecanoylamino)ethoxy)ethoxy]acetylamino}hexanoylamino)ethoxy]ethoxy}) acetyl-[$Aib^{8,22,35}$]GLP-1(7-37) amide,

$N^{\epsilon 38}$-(2-(2-(2-(4-(hexadecanoylamino)-4(S)-carboxybutyrylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,$Arg^{26,34}$,Lys^{38}]GLP-1(7-37),

$N^{\epsilon 38}$-(2-{2-[4-[4-(4-Amino-9,10-dioxo-3-sulfo-9,10-dihydro-anthracen-1-ylamino)-2-sulfo-phenylamino]-6-(2-sulfo-phenylamino)-[1,3,5]triazin-2-ylamino]-ethoxy}-ethoxy)-acetyl)-[$Aib^{8,22,35}$,Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$-({2-[2-(2-{2-[2-(2-{2-[2-(15-carboxypentadecanoylamino)-ethoxy]ethoxy}acetylamino)ethoxy]ethoxy}acetyl amino)ethoxy]ethoxy}acetyl)- [$Aib^{8,22,35}$, Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$-([2-(2-{3-[2,5-dioxo-3-(15-carboxypentadecylsulfanyl)-pyrrolidin-1-yl]-propionylamino}ethoxy)ethoxy)acetyl]-[$D\text{-}Ala^{8}$,$Lys^{37,38}$]GLP-1-[7-37] amide,

$N^{\epsilon 38}$-(2-(2-(2-(11-(oxalylamino)undecanoylamino)ethoxy)ethoxy)acetyl-)) [$Aib^{8,22,35}$, Ala^{37},Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$({2-[2-(2-{2-[2-(2-(15-carboxy-pentadecanoylamino)-ethoxy]ethoxy}acetylamino)ethoxy]ethoxy}acetyl)[$Aib^{8,22,35}$,Ala^{37},Lys^{38}]GLP-1(7-37) amide,

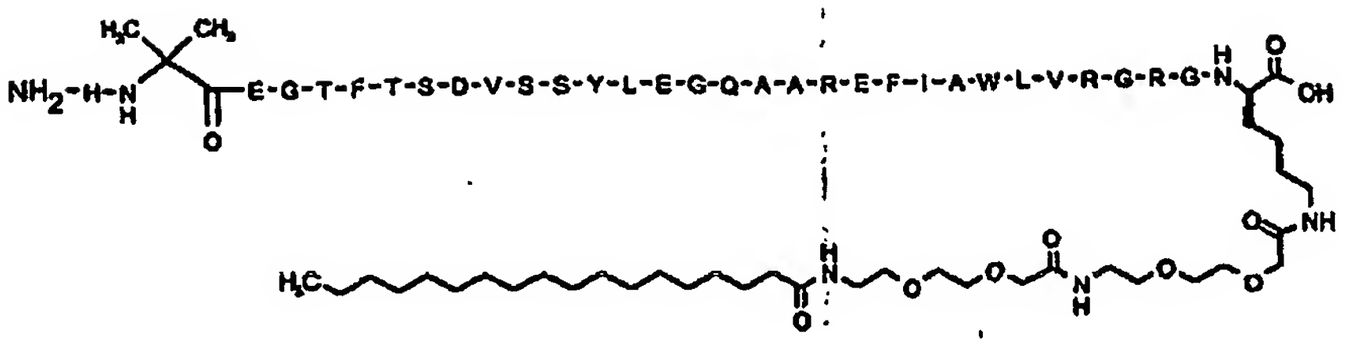
$N^{\epsilon 38}$-((2-{2-[11-(5-Dimethylaminonaphthalene-1-sulfonylamino)undecanoylamino]ethoxy}ethoxy)acetyl [$Aib^{8,22,35}$,Ala^{37},Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$([2-(2-{2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetylamino}ethoxy)ethoxy]acetyl) [$Aib^{8,22,35}$,Ala^{37},Lys^{38}]GLP-1(7-37) amide,

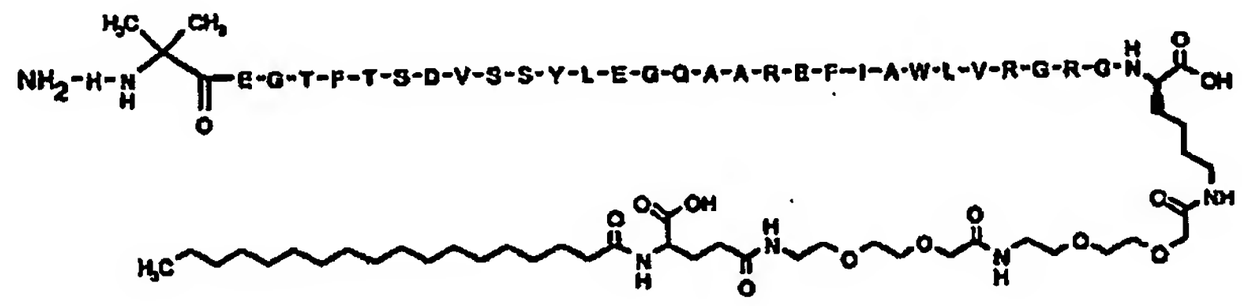
$N^{\epsilon 38}$-2-(2-(2-(octadecanoylamino)ethoxy)ethoxy)acetyl [Aib^{8},$Arg^{26,34}$,$Glu^{22,23,30}$,Lys^{38}]GLP-1 (7-37) amide,

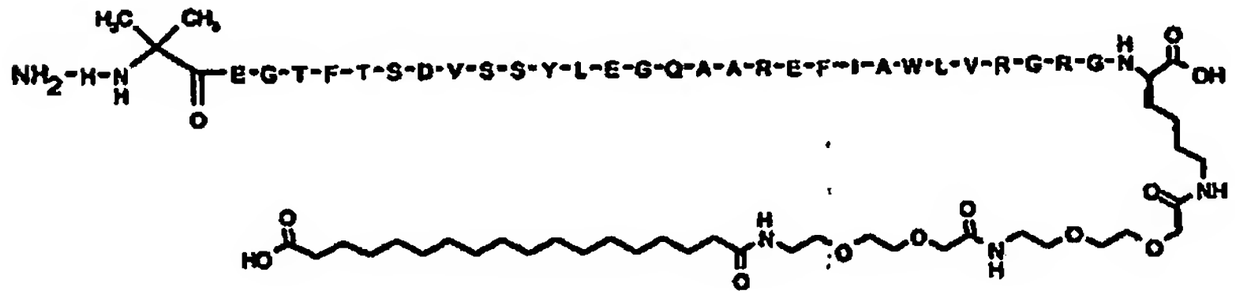
$N^{\epsilon 38}$-2-(2-(2-(eicosanoylamino)ethoxy)ethoxy)acetyl [Aib^{8},$Arg^{26,34}$,$Glu^{22,23,30}$,Lys^{38}]GLP-1(7-37) amide,

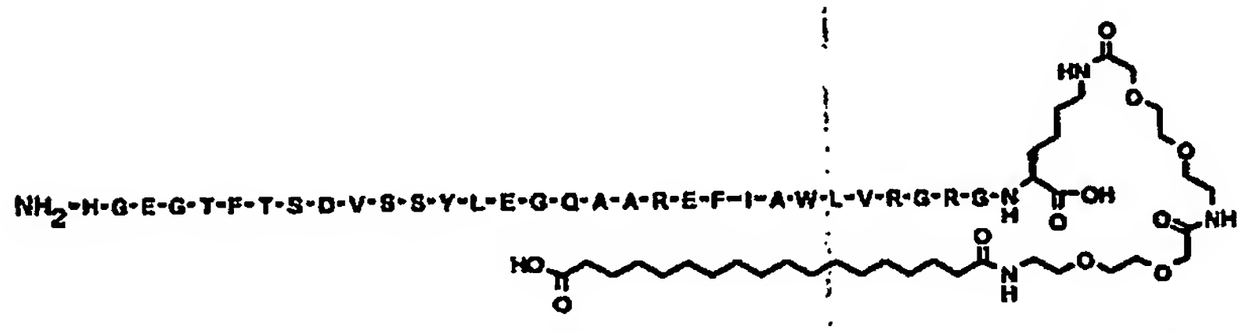
$N^{\epsilon 38}$({2-[2-(2-{2-[2-(4-Carboxy-4-octadecanoylamino-butyrylamino)ethoxy]ethoxy}acetylamino)ethoxy]ehoxy}-acetic acid)[Gly^{8},$Arg^{26,34,38}$,Lys^{38}]GLP-1(7-37),



$N^{\epsilon 38}$-(2-(2-(2-(gamma-glutamyl(N-alfa-hexadecanoyl))aminoethoxy)ethoxy)acetyl)[Aib^{8},Lys^{38}]GLP-1(7-37),







$N^{\epsilon 38}$-(2-(2-(2-(2-(2-(2-(gamma-glutamyl(N-alfa-hexadecanoyl))aminoethoxy)ethoxy)acetyl)aminoethoxy)ethoxy)acetyl)[Aib^{8},Lys^{38}]GLP-1(7-38),

$N^{\epsilon 37}$-(C12oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(C14oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(C16oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(C18oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide and

$N^{\epsilon 37}$-(C20oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide.

In another embodiment the therapeutic polypeptide is a GLP-2 peptide.

In another embodiment the GLP-2 peptide is a DPPIV-protected GLP-2 peptide.

In another embodiment the GLP-2 peptide is Gly^{2}-GLP-2(1-33).

In yet another embodiment the GLP-2 peptide is $Lys^{17}Arg^{30}$-GLP-2(1-33).

In another embodiment of the invention the therapeutic polypeptide is human insulin or an analogue thereof.

In another embodiment of the invention the therapeutic polypeptide is selected from the group consisting of Asp^{B28}-human insulin, Lys^{B28},Pro^{B29}-human insulin, Lys^{B3},Glu^{B29}-human insulin, Gly^{A21},Arg^{B31},Arg^{B32}-human insulin and des(B30) human insulin.

In another embodiment of the invention the therapeutic polypeptide is human growth hormone or an analogue thereof.

In another embodiment of the invention the therapeutic polypeptide is parathyroid hormone or an analogue thereof.

In another embodiment of the invention the therapeutic polypeptide is human follicle stimulating hormone or an analogue thereof.

In another embodiment of the invention the therapeutic polypeptide has a molar weight of less than 100 kDa, less than 50 kDa, or less than 10 kDa.

In another embodiment of the invention the therapeutic polypeptide is selected from the group consisting of a growth factor such as platelet-derived growth factor (PDGF), transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), a somatomedin such as insulin growth factor I (IGF-I), insulin growth factor II (IFG-II), erythropoietin (EPO), thrombopoietin (TPO) or angiopoietin, interferon, pro-urokinase, urokinase, tissue plasminogen activator (t-PA), plasminogen activator inhibitor 1, plasminogen activator inhibitor 2, von Willebrandt factor, a cytokine, e.g. an interleukin such as interleukin (IL) 1, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-9, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-20 or IL-21, a colony stimulating factor (CFS) such as GM-CSF, stem cell factor, a tumor necrosis factor such as TNF-α, lymphotoxin-α, lymphotoxin-β, CD40L, or CD30L, a protease inhibitor e.g. aprotinin, an enzyme such as superoxide dismutase, asparaginase, arginase, arginine deaminase, adenosine deaminase, ribonuclease, catalase, uricase, bilirubin oxidase, trypsin, papain, alkaline phosphatase, β-glucoronidase, purine nucleoside phosphorylase or batroxobin, an opioid, e.g. endorphins, enkephalins or non-natural opioids, a hormone or neuropeptide, e.g. calcitonin, glucagon, gastrins, adrenocorticotropic hormone (ACTH), cholecystokinins, lutenizing hormone, gonadotropin-releassing hormone, chorionic gonadotropin, corticotrophin-releasing factor, vasopressin, oxytocin, antidiuretic hormones, thyroid-stimulating hormone, thyrotropin-releasing hormone, relaxin, prolactin, peptide YY, neuropeptide Y, pancreastic polypeptide, leptin, CART (cocaine and amphetamine regulated transcript), a CART related peptide, perilipin, melanocortins (melanocyte-stimulating hormones) such as MC-4, melanin-concentrating hormones, natriuretic peptides, adrenomedullin, endothelin, secretin, amylin, vasoactive intestinal peptide (VIP), pituary adenylate cyclase acti-

vating polypeptide (PACAP), bombesin, bombesin-like peptides, thymosin, heparin-binding protein, soluble CD4, hypothalmic releasing factor, melanotonins and analogues thereof.

In another aspect the present invention relates to a pharmaceutical composition comprising a compound according to the invention, and a pharmaceutically acceptable excipient.

In one embodiment the pharmaceutical composition is suited for parenteral administration.

In another aspect the present invention relates to the use of a compound according to the invention for the preparation of a medicament.

In one embodiment of the invention a compound according to the invention wherein the therapeutic polypeptide is a GLP-1 peptide is used for the preparation of a medicament for the treatment or prevention of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, hypertension, syndrome X, dyslipidemia, cognitive disorders, atherosclerosis, myocardial infarction, coronary heart disease and other cardiovascular disorders, stroke, inflammatory bowel syndrome, dyspepsia and gastric ulcers.

In another embodiment of the invention a compound according to the invention wherein the therapeutic polypeptide is a GLP-1 peptide is used for the preparation of a medicament for delaying or preventing disease progression in type 2 diabetes.

In another embodiment of the invention a compound according to the invention wherein the therapeutic polypeptide is a GLP-1 peptide is used for the preparation of a medicament for decreasing food intake, decreasing β-cell apoptosis, increasing β-cell funtion and β-cell mass, and/or for restoring glucose sensitivity to β-cells.

In another embodiment of the invention a compound according to the invention wherein the therapeutic polypeptide is a GLP-2 peptide is used for the preparation of a medicament for the treatment of small bowel syndrome, inflammatory bowel syndrome or Crohns disease.

In another embodiment of the invention a compound according to the invention wherein the therapeutic polypeptide is an insulin peptide is used for the preparation of a medicament for the treatment or prevention of hyperglycemia, type 1 diabetes, type 2 diabetes or β-cell deficiency.

The therapeutic polypeptide can be produced by a method which comprises culturing a host cell containing a DNA sequence encoding the polypeptide and capable of expressing the poly-

peptide in a suitable nutrient medium under conditions permitting the expression of the peptide, after which the resulting peptide is recovered from the culture.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.* in catalogues of the American Type Culture Collection). The peptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, *e.g.* ammonium sulphate, purification by a variety of chromatographic procedures, *e.g.* ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like, dependent on the type of peptide in question.

The DNA sequence encoding the therapeutic polypeptide may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the polypeptide by hybridisation using synthetic oligonucleotide probes in accordance with standard techniques (see, for example, Sambrook, J, Fritsch, EF and Maniatis, T, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989). The DNA sequence encoding the polypeptide may also be prepared synthetically by established standard methods, *e.g.* the phosphoamidite method described by Beaucage and Caruthers, *Tetrahedron Letters* **22** (1981), 1859 - 1869, or the method described by Matthes *et al.*, *EMBO Journal* **3** (1984), 801 - 805. The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki *et al.*, *Science* **239** (1988), 487 - 491.

The DNA sequence may be inserted into any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, *i.e.* a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.* a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the peptide is operably linked to additional segments required for transcription of the DNA, such as a pro-

moter. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the peptide of the invention in a variety of host cells are well known in the art, cf. for instance Sambrook *et al.*, *supra*.

The DNA sequence encoding the peptide may also, if necessary, be operably connected to a suitable terminator, polyadenylation signals, transcriptional enhancer sequences, and translational enhancer sequences. The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, *e.g.* a gene the product of which complements a defect in the host cell or one which confers resistance to a drug, *e.g.* ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate.

To direct a parent peptide of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the peptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that normally associated with the peptide or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook *et al.*., *supra*).

The host cell into which the DNA sequence or the recombinant vector is introduced may be any cell which is capable of producing the present peptide and includes bacteria, yeast, fungi and higher eukaryotic cells. Examples of suitable host cells well known and used in the art are, without limitation, *E. coli*, *Saccharomyces cerevisiae*, or mammalian BHK or CHO cell lines.

Examples of compounds which can be useful as GLP-1 moieties according to the present invention are described in International Patent Application No. WO 87/06941 (The General Hos-

pital Corporation) which relates to a peptide fragment which comprises GLP-1(7-37) and functional derivatives thereof and to its use as an insulinotropic agent.

Further GLP-1 analogues are described in International Patent Application No. 90/11296 (The General Hospital Corporation) which relates to peptide fragments which comprise GLP-1(7-36) and functional derivatives thereof and have an insulinotropic activity which exceeds the insulinotropic activity of GLP-1(1-36) or GLP-1(1-37) and to their use as insulinotropic agents.

International Patent Application No. 91/11457 (Buckley *et al.*.) discloses analogues of the active GLP-1 peptides 7-34, 7-35, 7-36, and 7-37 which can also be useful as GLP-1 moieties according to the present invention.

Pharmaceutical compositions

Pharmaceutical compositions containing a compound according to the present invention may be prepared by conventional techniques, *e.g.* as described in Remington's *Pharmaceutical Sciences*, 1985 or in Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

One object of the present invention is to provide a pharmaceutical formulation comprising a compound according to the present invention which is present in a concentration from about 0.1 mg/ml to about 25 mg/ml, and wherein said formulation has a pH from 2.0 to 10.0. The formulation may further comprise a buffer system, preservative(s), isotonicity agent(s), chelating agent(s), stabilizers and surfactants. In one embodiment of the invention the pharmaceutical formulation is an aqueous formulation, i.e. formulation comprising water. Such formulation is typically a solution or a suspension. In a further embodiment of the invention the pharmaceutical formulation is an aqueous solution. The term "aqueous formulation" is defined as a formulation comprising at least 50 %w/w water. Likewise, the term "aqueous solution" is defined as a solution comprising at least 50 %w/w water, and the term "aqueous suspension" is defined as a suspension comprising at least 50 %w/w water.

In another embodiment the pharmaceutical formulation is a freeze-dried formulation, whereto the physician or the patient adds solvents and/or diluents prior to use.

In another embodiment the pharmaceutical formulation is a dried formulation (e.g. freeze-dried or spray-dried) ready for use without any prior dissolution.

In a further aspect the invention relates to a pharmaceutical formulation comprising an aqueous solution of a compound according to the present invention, and a buffer,

wherein said compound is present in a concentration from 0.1 mg/ml or above, and wherein said formulation has a pH from about 2.0 to about 10.0.

In a another embodiment of the invention the pH of the formulation is selected from the list consisting of 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, and 10.0. Preferably, the pH of the formulation is at least 1 pH unit from the isoelectric point of the compound according to the present invention, even more preferable the pH of the formulation is at least 2 pH unit from the isoelectric point of the compound according to the present invention.

In a further embodiment of the invention the buffer is selected from the group consisting of sodium acetate, sodium carbonate, citrate, glycylglycine, histidine, glycine, lysine, arginine, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium phosphate, and tris(hydroxymethyl)-aminomethane, hepes, bicine, tricine, malic acid, succinate, maleic acid, fumaric acid, tartaric acid, aspartic acid or mixtures thereof. Each one of these specific buffers constitutes an alternative embodiment of the invention.

In a further embodiment of the invention the formulation further comprises a pharmaceutically acceptable preservative. In a further embodiment of the invention the preservative is selected from the group consisting of phenol, o-cresol, m-cresol, p-cresol, methyl p-hydroxybenzoate, propyl p-hydroxybenzoate, 2-phenoxyethanol, butyl p-hydroxybenzoate, 2-phenylethanol, benzyl alcohol, chlorobutanol, and thiomerosal, bronopol, benzoic acid, imidurea, chlorohexidine, sodium dehydroacetate, chlorocresol, ethyl p-hydroxybenzoate, benzethonium chloride, chlorphenesine (3p-chlorphenoxypropane-1,2-diol) or mixtures thereof. In a further embodiment of the invention the preservative is present in a concentration from 0.1 mg/ml to 20 mg/ml. In a further embodiment of the invention the preservative is present in a concentration from 0.1 mg/ml to 5 mg/ml. In a further embodiment of the invention the preservative is present in a concentration from 5 mg/ml to 10 mg/ml. In a further embodiment of the invention the preservative is present in a concentration from 10 mg/ml to 20 mg/ml. Each one of these specific preservatives constitutes an alternative embodiment of the invention. The use of a preservative in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

In a further embodiment of the invention the formulation further comprises an isotonic agent. In a further embodiment of the invention the isotonic agent is selected from the group consisting of a salt (e.g. sodium chloride), a sugar or sugar alcohol, an amino acid (e.g. L-glycine, L-histidine, arginine, lysine, isoleucine, aspartic acid, tryptophan, threonine), an alditol (e.g. glycerol (glycerine), 1,2-propanediol (propyleneglycol), 1,3-propanediol, 1,3-butanediol) polyethyleneglycol (e.g. PEG400), or mixtures thereof. Any sugar such as mono-, di-, or polysaccharides, or water-soluble glucans, including for example fructose, glucose, mannose, sorbose, xylose, maltose, lactose, sucrose, trehalose, dextran, pullulan, dextrin, cyclodextrin, soluble starch, hydroxyethyl starch and carboxymethylcellulose-Na may be used. In one embodiment the sugar additive is sucrose. Sugar alcohol is defined as a C4-C8 hydrocarbon having at least one --OH group and includes, for example, mannitol, sorbitol, inositol, galactititol, dulcitol, xylitol, and arabitol. In one embodiment the sugar alcohol additive is mannitol. The sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to the amount used, as long as the sugar or sugar alcohol is soluble in the liquid preparation and does not adversely effect the stabilizing effects achieved using the methods of the invention. In one embodiment, the sugar or sugar alcohol concentration is between about 1 mg/ml and about 150 mg/ml. In a further embodiment of the invention the isotonic agent is present in a concentration from 1 mg/ml to 50 mg/ml. In a further embodiment of the invention the isotonic agent is present in a concentration from 1 mg/ml to 7 mg/ml. In a further embodiment of the invention the isotonic agent is present in a concentration from 8 mg/ml to 24 mg/ml. In a further embodiment of the invention the isotonic agent is present in a concentration from 25 mg/ml to 50 mg/ml. Each one of these specific isotonic agents constitutes an alternative embodiment of the invention. The use of an isotonic agent in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

In a further embodiment of the invention the formulation further comprises a chelating agent. In a further embodiment of the invention the chelating agent is selected from salts of ethylenediaminetetraacetic acid (EDTA), citric acid, and aspartic acid, and mixtures thereof. In a further embodiment of the invention the chelating agent is present in a concentration from 0.1mg/ml to 5mg/ml. In a further embodiment of the invention the chelating agent is present in a concentration from 0.1mg/ml to 2mg/ml. In a further embodiment of the invention the chelating agent is present in a concentration from 2mg/ml to 5mg/ml. Each one of these specific chelating agents constitutes an alternative embodiment of the invention. The use of a chelating agent in pharmaceutical compositions is well-known to the skilled person.

For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

In a further embodiment of the invention the formulation further comprises a stabiliser. The use of a stabilizer in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

More particularly, compositions of the invention are stabilized liquid pharmaceutical compositions whose therapeutically active components include a polypeptide that possibly exhibits aggregate formation during storage in liquid pharmaceutical formulations. By "aggregate formation" is intended a physical interaction between the polypeptide molecules that results in formation of oligomers, which may remain soluble, or large visible aggregates that precipitate from the solution. By "during storage" is intended a liquid pharmaceutical composition or formulation once prepared, is not immediately administered to a subject. Rather, following preparation, it is packaged for storage, either in a liquid form, in a frozen state, or in a dried form for later reconstitution into a liquid form or other form suitable for administration to a subject. By "dried form" is intended the liquid pharmaceutical composition or formulation is dried either by freeze drying (i.e., lyophilization; see, for example, Williams and Polli (1984) J. Parenteral Sci. Technol. 38:48-59), spray drying (see Masters (1991) in Spray-Drying Handbook (5th ed; Longman Scientific and Technical, Essez, U.K.), pp. 491-676; Broadhead et al. (1992) Drug Devel. Ind. Pharm. 18:1169-1206; and Mumenthaler et al. (1994) Pharm. Res. 11:12-20), or air drying (Carpenter and Crowe (1988) Cryobiology 25:459-470; and Roser (1991) Biopharm. 4:47-53). Aggregate formation by a polypeptide during storage of a liquid pharmaceutical composition can adversely affect biological activity of that polypeptide, resulting in loss of therapeutic efficacy of the pharmaceutical composition. Furthermore, aggregate formation may cause other problems such as blockage of tubing, membranes, or pumps when the polypeptide-containing pharmaceutical composition is administered using an infusion system.

The pharmaceutical compositions of the invention may further comprise an amount of an amino acid base sufficient to decrease aggregate formation by the polypeptide during storage of the composition. By "amino acid base" is intended an amino acid or a combination of amino acids, where any given amino acid is present either in its free base form or in its salt form. Where a combination of amino acids is used, all of the amino acids may be present in their free base forms, all may be present in their salt forms, or some may be present in their free base forms while others are present in their salt forms. In one embodiment, amino acids to use in preparing the compositions of the invention are those carrying a

charged side chain, such as arginine, lysine, aspartic acid, and glutamic acid. Any stereoisomer (i.e., L, D, or DL isomer) of a particular amino acid (e.g. glycine, methionine, histidine, imidazole, arginine, lysine, isoleucine, aspartic acid, tryptophan, threonine and mixtures thereof) or combinations of these stereoisomers, may be present in the pharmaceutical compositions of the invention so long as the particular amino acid is present either in its free base form or its salt form. In one embodiment the L-stereoisomer is used. Compositions of the invention may also be formulated with analogues of these amino acids. By "amino acid analogue" is intended a derivative of the naturally occurring amino acid that brings about the desired effect of decreasing aggregate formation by the polypeptide during storage of the liquid pharmaceutical compositions of the invention. Suitable arginine analogues include, for example, aminoguanidine, ornithine and N-monoethyl L-arginine, suitable methionine analogues include ethionine and buthionine and suitable cystein analogues include S-methyl-L cystein. As with the other amino acids, the amino acid analogues are incorporated into the compositions in either their free base form or their salt form. In a further embodiment of the invention the amino acids or amino acid analogues are used in a concentration, which is sufficient to prevent or delay aggregation of the protein.

In a further embodiment of the invention methionine (or other sulphuric amino acids or amino acid analogous) may be added to inhibit oxidation of methionine residues to methionine sulfoxide when the polypeptide acting as the therapeutic agent is a polypeptide comprising at least one methionine residue susceptible to such oxidation. By "inhibit" is intended minimal accumulation of methionine oxidized species over time. Inhibiting methionine oxidation results in greater retention of the polypeptide in its proper molecular form. Any stereoisomer of methionine (L, D, or DL isomer) or combinations thereof can be used. The amount to be added should be an amount sufficient to inhibit oxidation of the methionine residues such that the amount of methionine sulfoxide is acceptable to regulatory agencies. Typically, this means that the composition contains no more than about 10% to about 30% methionine sulfoxide. Generally, this can be achieved by adding methionine such that the ratio of methionine added to methionine residues ranges from about 1:1 to about 1000:1, such as 10:1 to about 100:1.

In a further embodiment of the invention the formulation further comprises a stabiliser selected from the group of high molecular weight polymers or low molecular compounds. In a further embodiment of the invention the stabilizer is selected from polyethylene glycol (e.g. PEG 3350), polyvinylalcohol (PVA), polyvinylpyrrolidone, carboxy-/hydroxycellulose or derivates thereof (e.g. HPC, HPC-SL, HPC-L and HPMC), cyclodextrins, sulphur-containing substances as monothioglycerol, thioglycolic acid and 2-methylthioethanol, and different

salts (e.g. sodium chloride). Each one of these specific stabilizers constitutes an alternative embodiment of the invention.

The pharmaceutical compositions may also comprise additional stabilizing agents, which further enhance stability of a therapeutically active polypeptide therein. Stabilizing agents of particular interest to the present invention include, but are not limited to, methionine and EDTA, which protect the polypeptide against methionine oxidation, and a nonionic surfactant, which protects the polypeptide against aggregation associated with freeze-thawing or mechanical shearing.

In a further embodiment of the invention the formulation further comprises a surfactant. In a further embodiment of the invention the surfactant is selected from a detergent, ethoxylated castor oil, polyglycolyzed glycerides, acetylated monoglycerides, sorbitan fatty acid esters, polyoxypropylene-polyoxyethylene block polymers (eg. poloxamers such as Pluronic® F68, poloxamer 188 and 407, Triton X-100), polyoxyethylene sorbitan fatty acid esters, polyoxyethylene and polyethylene derivatives such as alkylated and alkoxylated derivatives (tweens, e.g. Tween-20, Tween-40, Tween-80 and Brij-35), monoglycerides or ethoxylated derivatives thereof, diglycerides or polyoxyethylene derivatives thereof, alcohols, glycerol, lecitins and phospholipids (eg. phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, diphosphatidyl glycerol and sphingomyelin), derivates of phospholipids (eg. dipalmitoyl phosphatidic acid) and lysophospholipids (eg. palmitoyl lysophosphatidyl-L-serine and 1-acyl-sn-glycero-3-phosphate esters of ethanolamine, choline, serine or threonine) and alkyl, alkoxyl (alkyl ester), alkoxy (alkyl ether)- derivatives of lysophosphatidyl and phosphatidylcholines, e.g. lauroyl and myristoyl derivatives of lysophosphatidylcholine, dipalmitoylphosphatidylcholine, and modifications of the polar head group, that is cholines, ethanolamines, phosphatidic acid, serines, threonines, glycerol, inositol, and the positively charged DODAC, DOTMA, DCP, BISHOP, lysophosphatidylserine and lysophosphatidylthreonine, and glycerophospholipids (eg. cephalins), glyceroglycolipids (eg. galactopyransoide), sphingoglycolipids (eg. ceramides, gangliosides), dodecylphosphocholine, hen egg lysolecithin, fusidic acid derivatives- (e.g. sodium tauro-dihydrofusidate etc.), long-chain fatty acids and salts thereof C6-C12 (eg. oleic acid and caprylic acid), acylcarnitines and derivatives, N^{α}-acylated derivatives of lysine, arginine or histidine, or side-chain acylated derivatives of lysine or arginine, N^{α}-acylated derivatives of dipeptides comprising any combination of lysine, arginine or histidine and a neutral or acidic amino acid, N^{α}-acylated derivative of a tripeptide comprising any combination of a neutral amino acid and two charged amino acids, DSS (docusate sodium, CAS registry no [577-11-7]), docusate

calcium, CAS registry no [128-49-4]), docusate potassium, CAS registry no [7491-09-0]), SDS (sodium dodecyl sulfate or sodium lauryl sulfate), sodium caprylate, cholic acid or derivatives thereof, bile acids and salts thereof and glycine or taurine conjugates, ursodeoxycholic acid, sodium cholate, sodium deoxycholate, sodium taurocholate, sodium glycocholate, N-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, anionic (alkyl-aryl-sulphonates) monovalent surfactants, zwitterionic surfactants (e.g. N-alkyl-N,N-dimethylammonio-1-propanesulfonates, 3-cholamido-1-propyldimethylammonio-1-propanesulfonate, cationic surfactants (quarternary ammonium bases) (e.g. cetyl-trimethylammonium bromide, cetylpyridinium chloride), non-ionic surfactants (eg. Dodecyl β-D-glucopyranoside), poloxamines (eg. Tetronic's), which are tetrafunctional block copolymers derived from sequential addition of propylene oxide and ethylene oxide to ethylenediamine, or the surfactant may be selected from the group of imidazoline derivatives, or mixtures thereof. Each one of these specific surfactants constitutes an alternative embodiment of the invention.

The use of a surfactant in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

It is possible that other ingredients may be present in the peptide pharmaceutical formulation of the present invention. Such additional ingredients may include wetting agents, emulsifiers, antioxidants, bulking agents, tonicity modifiers, chelating agents, metal ions, oleaginous vehicles, proteins (e.g., human serum albumin, gelatin or proteins) and a zwitterion (e.g., an amino acid such as betaine, taurine, arginine, glycine, lysine and histidine). Such additional ingredients, of course, should not adversely affect the overall stability of the pharmaceutical formulation of the present invention.

Pharmaceutical compositions containing a compound according to the present invention may be administered to a patient in need of such treatment at several sites, for example, at topical sites, for example, skin and mucosal sites, at sites which bypass absorption, for example, administration in an artery, in a vein, in the heart, and at sites which involve absorption, for example, administration in the skin, under the skin, in a muscle or in the abdomen.

Administration of pharmaceutical compositions according to the invention may be through several routes of administration, for example, lingual, sublingual, buccal, in the mouth, oral, in the stomach and intestine, nasal, pulmonary, for example, through the bronchioles and alveoli or a combination thereof, epidermal, dermal, transdermal, vaginal, rectal,

ocular, for examples through the conjunctiva, uretal, and parenteral to patients in need of such a treatment.

Compositions of the current invention may be administered in several dosage forms, for example, as solutions, suspensions, emulsions, microemulsions, multiple emulsion, foams, salves, pastes, plasters, ointments, tablets, coated tablets, rinses, capsules, for example, hard gelatine capsules and soft gelatine capsules, suppositories, rectal capsules, drops, gels, sprays, powder, aerosols, inhalants, eye drops, ophthalmic ointments, ophthalmic rinses, vaginal pessaries, vaginal rings, vaginal ointments, injection solution, in situ transforming solutions, for example in situ gelling, in situ setting, in situ precipitating, in situ crystallization, infusion solution, and implants.

Compositions of the invention may further be compounded in, or attached to, for example through covalent, hydrophobic and electrostatic interactions, a drug carrier, drug delivery system and advanced drug delivery system in order to further enhance stability of the compound, increase bioavailability, increase solubility, decrease adverse effects, achieve chronotherapy well known to those skilled in the art, and increase patient compliance or any combination thereof. Examples of carriers, drug delivery systems and advanced drug delivery systems include, but are not limited to, polymers, for example cellulose and derivatives, polysaccharides, for example dextran and derivatives, starch and derivatives, poly(vinyl alcohol), acrylate and methacrylate polymers, polylactic and polyglycolic acid and block copolymers thereof, polyethylene glycols, carrier proteins, for example albumin, gels, for example, thermogelling systems, for example block co-polymeric systems well known to those skilled in the art, micelles, liposomes, microspheres, nanoparticulates, liquid crystals and dispersions thereof, L2 phase and dispersions there of, well known to those skilled in the art of phase behaviour in lipid-water systems, polymeric micelles, multiple emulsions, self-emulsifying, self-microemulsifying, cyclodextrins and derivatives thereof, and dendrimers.

Compositions of the current invention are useful in the formulation of solids, semi-solids, powder and solutions for pulmonary administration of the compound, using, for example a metered dose inhaler, dry powder inhaler and a nebulizer, all being devices well known to those skilled in the art.

Compositions of the current invention are specifically useful in the formulation of controlled, sustained, protracting, retarded, and slow release drug delivery systems. More specifically, but not limited to, compositions are useful in formulation of parenteral controlled release and sustained release systems (both systems leading to a many-fold reduction in number of administrations), well known to those skilled in the art. Even more preferably, are controlled release and sustained release systems administered subcutaneous. Without limiting the scope of the invention, examples of useful controlled release system and composi-

tions are hydrogels, oleaginous gels, liquid crystals, polymeric micelles, microspheres, nanoparticles,

Methods to produce controlled release systems useful for compositions of the current invention include, but are not limited to, crystallization, condensation, co-cystallization, precipitation, co-precipitation, emulsification, dispersion, high pressure homogenization, encapsulation, spray drying, microencapsulation, coacervation, phase separation, solvent evaporation to produce microspheres, extrusion and supercritical fluid processes. General reference is made to Handbook of Pharmaceutical Controlled Release (Wise, D.L., ed. Marcel Dekker, New York, 2000) and Drug and the Pharmaceutical Sciences vol. 99: Protein Formulation and Delivery (MacNally, E.J., ed. Marcel Dekker, New York, 2000).

Parenteral administration may be performed by subcutaneous, intramuscular, intraperitoneal or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a solution or suspension for the administration of the compound according to the present invention in the form of a nasal or pulmonal spray. As a still further option, the pharmaceutical compositions containing the compound of the invention can also be adapted to transdermal administration, *e.g.* by needle-free injection or from a patch, optionally an iontophoretic patch, or transmucosal, *e.g.* buccal, administration.

The term "stabilized formulation" refers to a formulation with increased physical stability, increased chemical stability or increased physical and chemical stability.

The term "physical stability" of the protein formulation as used herein refers to the tendency of the protein to form biologically inactive and/or insoluble aggregates of the protein as a result of exposure of the protein to thermo-mechanical stresses and/or interaction with interfaces and surfaces that are destabilizing, such as hydrophobic surfaces and interfaces. Physical stability of the aqueous protein formulations is evaluated by means of visual inspection and/or turbidity measurements after exposing the formulation filled in suitable containers (e.g. cartridges or vials) to mechanical/physical stress (e.g. agitation) at different temperatures for various time periods. Visual inspection of the formulations is performed in a sharp focused light with a dark background. The turbidity of the formulation is characterized by a visual score ranking the degree of turbidity for instance on a scale from 0 to 3 (a formulation showing no turbidity corresponds to a visual score 0, and a formulation showing visual turbidity in daylight corresponds to visual score 3). A formulation is classified physical unstable with respect to protein aggregation, when it shows visual turbidity in daylight. Alternatively, the turbidity of the formulation can be evaluated by simple turbidity measurements well-known to the skilled person. Physical stability of the aqueous protein formulations can also be evaluated by using a spec-

troscopic agent or probe of the conformational status of the protein. The probe is preferably a small molecule that preferentially binds to a non-native conformer of the protein. One example of a small molecular spectroscopic probe of protein structure is Thioflavin T. Thioflavin T is a fluorescent dye that has been widely used for the detection of amyloid fibrils. In the presence of fibrils, and perhaps other protein configurations as well, Thioflavin T gives rise to a new excitation maximum at about 450 nm and enhanced emission at about 482 nm when bound to a fibril protein form. Unbound Thioflavin T is essentially non-fluorescent at the wavelengths.

Other small molecules can be used as probes of the changes in protein structure from native to non-native states. For instance the "hydrophobic patch" probes that bind preferentially to exposed hydrophobic patches of a protein. The hydrophobic patches are generally buried within the tertiary structure of a protein in its native state, but become exposed as a protein begins to unfold or denature. Examples of these small molecular, spectroscopic probes are aromatic, hydrophobic dyes, such as antrhacene, acridine, phenanthroline or the like. Other spectroscopic probes are metal-amino acid complexes, such as cobalt metal complexes of hydrophobic amino acids, such as phenylalanine, leucine, isoleucine, methionine, and valine, or the like.

The term "chemical stability" of the protein formulation as used herein refers to chemical covalent changes in the protein structure leading to formation of chemical degradation products with potential less biological potency and/or potential increased immunogenic properties compared to the native protein structure. Various chemical degradation products can be formed depending on the type and nature of the native protein and the environment to which the protein is exposed. Elimination of chemical degradation can most probably not be completely avoided and increasing amounts of chemical degradation products is often seen during storage and use of the protein formulation as well-known by the person skilled in the art. Most proteins are prone to deamidation, a process in which the side chain amide group in glutaminyl or asparaginyl residues is hydrolysed to form a free carboxylic acid. Other degradations pathways involves formation of high molecular weight transformation products where two or more protein molecules are covalently bound to each other through transamidation and/or disulfide interactions leading to formation of covalently bound dimer, oligomer and polymer degradation products (*Stability of Protein Pharmaceuticals, Ahern. T.J. & Manning M.C., Plenum Press, New York 1992*). Oxidation (of for instance methionine residues) can be mentioned as another variant of chemical degradation. The chemical stability of the protein formulation can be evaluated by measuring the amount of the chemical degradation products at various time-points after exposure to different environmental condi-

tions (the formation of degradation products can often be accelerated by for instance increasing temperature). The amount of each individual degradation product is often determined by separation of the degradation products depending on molecule size and/or charge using various chromatography techniques (e.g. SEC-HPLC and/or RP-HPLC).

Hence, as outlined above, a "stabilized formulation" refers to a formulation with increased physical stability, increased chemical stability or increased physical and chemical stability. In general, a formulation must be stable during use and storage (in compliance with recommended use and storage conditions) until the expiration date is reached.

In one embodiment of the invention the pharmaceutical formulation comprising the compound according to the present invention is stable for more than 6 weeks of usage and for more than 3 years of storage.

In another embodiment of the invention the pharmaceutical formulation comprising the compound according to the present invention is stable for more than 4 weeks of usage and for more than 3 years of storage.

In a further embodiment of the invention the pharmaceutical formulation comprising the compound according to the present invention is stable for more than 4 weeks of usage and for more than two years of storage.

In an even further embodiment of the invention the pharmaceutical formulation comprising the compound is stable for more than 2 weeks of usage and for more than two years of storage.

Pharmaceutical compositions containing a GLP-1 derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the GLP-1 derivative in the form of a nasal or pulmonal spray. As a still further option, the GLP-1 derivatives of the invention can also be administered transdermally, *e.g.* from a patch, optionally a iontophoretic patch, or transmucosally, *e.g.* bucally.

Thus, the injectable compositions of the GLP-1 derivative of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

According to one procedure, the GLP-1 derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, *e.g.* hydrochloric acid, or a base, *e.g.* aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Further to the above-mentioned components, solutions containing a GLP-1 derivative according to the present invention may also contain a surfactant in order to improve the solubility and/or the stability of the GLP-1 derivative.

A composition for nasal administration of certain peptides may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S) or in WO 93/18785.

According to one preferred embodiment of the present invention, the GLP-1 derivative is provided in the form of a composition suitable for administration by injection. Such a composition can either be an injectable solution ready for use or it can be an amount of a solid composition, *e.g.* a lyophilised product, which has to be dissolved in a solvent before it can be injected. The injectable solution preferably contains not less than about 2 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml of the GLP-1 derivative and, preferably, not more than about 100 mg/ml of the GLP-1 derivative.

The GLP-1 derivatives of this invention can be used in the treatment of various diseases. The particular GLP-1 derivative to be used and the optimal dose level for any patient will depend on the disease to be treated and on a variety of factors including the efficacy of the specific peptide derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case. It is recommended that the dosage of the GLP-1 derivative of this invention be determined for each individual patient by those skilled in the art.

In particular, it is envisaged that the GLP-1 derivative will be useful for the preparation of a medicament with a protracted profile of action for the treatment of non-insulin dependent diabetes mellitus and/or for the treatment of obesity.

In another aspect the present invention relates to the use of a compound according to the invention for the preparation of a medicament.

In one embodiment the present invention relates to the use of a compound according to the invention for the preparation of a medicament for the treatment of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, hypertension, syndrome X, dyslipidemia, β-cell apoptosis, β-cell deficiency, myocardial infarction, inflammatory bowel syndrome, dyspepsia, cognitive disorders, e.g. cognitive enhancing, neuroprotection, atheroschlerosis, coronary heart disease and other cardiovascular disorders.

In another embodiment the present invention relates to the use of a compound according to the invention for the preparation of a medicament for the treatment of small bowel syndrome, inflammatory bowel syndrome or Crohns disease.

In another embodiment the present invention relates to the use of a compound according to the invention for the preparation of a medicament for the treatment of hyperglycemia, type 1 diabetes, type 2 diabetes or β-cell deficiency.

The treatment with a compound according to the present invention may also be combined with combined with a second or more pharmacologically active substances, e.g. selected from antidiabetic agents, antiobesity agents, appetite regulating agents, antihypertensive agents, agents for the treatment and/or prevention of complications resulting from or associated with diabetes and agents for the treatment and/or prevention of complications and disorders resulting from or associated with obesity. In the present context the expression "antidiabetic agent" includes compounds for the treatment and/or prophylaxis of insulin resistance and diseases wherein insulin resistance is the pathophysiological mechanism.

Examples of these pharmacologically active substances are : Insulin, GLP-1 agonists, sulphonylureas (e.g. tolbutamide, glibenclamide, glipizide and gliclazide), biguanides e.g. metformin, meglitinides, glucosidase inhibitors (e.g. acorbose), glucagon antagonists, DPP-IV (dipeptidyl peptidase-IV) inhibitors, inhibitors of hepatic enzymes involved in stimulation of gluconeogenesis and/or glycogenolysis, glucose uptake modulators, thiazolidinediones such as troglitazone and ciglitazone, compounds modifying the lipid metabolism such as antihyperlipidemic agents as HMG CoA inhibitors (statins), compounds lowering food intake, RXR agonists and agents acting on the ATP-dependent potassium channel of the β-cells, e.g. glibenclamide, glipizide, gliclazide and repaglinide; Cholestyramine, colestipol, clofibrate, gemfibrozil, lovastatin, pravastatin, simvastatin, probucol, dextrothyroxine, neteglinide, repaglinide; β-blockers such as alprenolol, atenolol, timolol, pindolol, propranolol and metoprolol, ACE (angiotensin converting enzyme) inhibitors such as benazepril, captopril, enalapril, fosinopril, lisinopril, alatriopril, quinapril and ramipril, calcium channel blockers

such as nifedipine, felodipine, nicardipine, isradipine, nimodipine, diltiazem and verapamil, and α-blockers such as doxazosin, urapidil, prazosin and terazosin; CART (cocaine amphetamine regulated transcript) agonists, NPY (neuropeptide Y) antagonists, MC4 (melanocortin 4) agonists, orexin antagonists, TNF (tumor necrosis factor) agonists, CRF (corticotropin releasing factor) agonists, CRF BP (corticotropin releasing factor binding protein) antagonists, urocortin agonists, β3 agonists, MSH (melanocyte-stimulating hormone) agonists, MCH (melanocyte-concentrating hormone) antagonists, CCK (cholecystokinin) agonists, serotonin re-uptake inhibitors, serotonin and noradrenaline re-uptake inhibitors, mixed serotonin and noradrenergic compounds, 5HT (serotonin) agonists, bombesin agonists, galanin antagonists, growth hormone, growth hormone releasing compounds, TRH (thyreotropin releasing hormone) agonists, UCP 2 or 3 (uncoupling protein 2 or 3) modulators, leptin agonists, DA agonists (bromocriptin, doprexin), lipase/amylase inhibitors, RXR (retinoid X receptor) modulators, TR β agonists; histamine H3 antagonists.

It should be understood that any suitable combination of the compounds according to the invention with one or more of the above-mentioned compounds and optionally one or more further pharmacologically active substances are considered to be within the scope of the present invention.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

EXAMPLES

The following acronyms for commercially available chemicals are used:

DMF	:	N,N-Dimethylformamide.
DCC	:	N,N-Dicyclohexylcarbodiimide
NMP	:	N-Methyl-2-pyrrolidone.
EDPA	:	N-Ethyl-N,N-diisopropylamine.
EGTA	:	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

GTP Guanosine 5'-triphosphate.

TFA : Trifluoroacetic acid.

THF : Tetrahydrofuran

H-Glu(OH)-OBu^t: L-Glutamic acid α-tert-butyl ester

Cap-ONSu: Octanoic acid 2,5-dioxopyrrolidin-1-yl ester

Lau-ONSu: Dodecanoic acid 2,5-dioxopyrrolidin-1-yl ester

Myr-ONSu: Tetradecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

Pal-ONSu: Hexadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

Ste-ONSu Octadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

Abbreviations:

PDMS: Plasma Desorption Mass Spectrometry

MALDI-MS: Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry

HPLC: High Performance Liquid Chromatography

amu: atomic mass units

Lit-Glu(ONSu)-OBu^t: N$^\alpha$-Lithochoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Cap-Glu(ONSu)-OBu^t: N$^\alpha$-Octanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Cac-Glu(ONSu)-OBu^t: N$^\alpha$-Decanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Lau-Glu(ONSu)-OBu^t: N$^\alpha$-Dodecanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Myr-Glu(ONSu)-OBu^t: N$^\alpha$-Tetradecanoyl-L-glutamic acid α-t-butyl ester γ-2,5-dioxopyrrolidin-1-yl ester

Pal-Glu(ONSu)-OBu^t : N$^\alpha$-Hexadecanoyl-(L)-glutamic acid α-t-butyl-γ-2,5-dioxopyrrolidin-1-yl diester.

Ste-Glu(ONSu)-OBu^t : N$^\alpha$-Octadecanoyl-(L)-glutamic acid α-t-butyl-γ-2,5-dioxopyrrolidin-1-yl diester

Lau-β-Ala-ONSu: N$^\beta$-Dodecanoyl-β-alanine 2,5-dioxopyrrolidin-1-yl ester

Pal-β-Ala-ONSu: N$^\beta$-Hexadecanoyl-β-alanine 2,5-dioxopyrrolidin-1-yl ester

Lau-GABA-ONSu: N$^\gamma$-Dodecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Myr-GABA-ONSu: N$^\gamma$-Tetradecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Pal-GABA-ONSu: N$^\gamma$-Hexadecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Ste-GABA-ONSu: N$^\gamma$-Octadecanoyl-γ-aminobutyric acid 2,5-dioxopyrrolidin-1-yl ester

Pal-Isonip-ONSu: N-Hexadecanoyl-piperidine-4-carboxylic acid 2,5-dioxopyrrolidin-1-yl ester

Pal-Glu(OBu^t)-ONSu: N$^\alpha$-Hexadecanoyl-L-glutamic acid α-2,5-dioxopyrrolidin-1-yl ester γ-t-butyl ester

HOOC-$(CH_2)_8$-COONSu: ω–Carboxyheptanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{10}$-COONSu: ω–Carboxyundecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{12}$-COONSu: ω–Carboxytridecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{14}$-COONSu: ω–Carboxypentadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{16}$-COONSu: ω–Carboxyheptadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

HOOC-$(CH_2)_{18}$-COONSu: ω–Carboxynonadecanoic acid 2,5-dioxopyrrolidin-1-yl ester.

Analytical

Plasma Desorption Mass Spectrometry

Sample preparation:

The sample is dissolved in 0.1 % TFA/EtOH (1:1) at a concentration of 1 µg/µl. The sample solution (5-10 µl) is placed on a nitrocellulose target (Bio-ion AB, Uppsala, Sweden) and allowed to adsorb to the target surface for 2 minutes. The target is subsequently rinsed with 2x25 µl 0.1 % TFA and spin-dried. Finally, the nitrocellulose target is placed in a target carrousel and introduced into the mass spectrometer.

MS analysis:

PDMS analysis was carried out using a Bio-ion 20 time-of flight instrument (Bio-ion Nordic AB, Uppsala, Sweden). An acceleration voltage of 15 kV was applied and molecular ions formed by bombardment of the nitrocellulose surface with 252-Cf fission fragments were accelerated towards a stop detector. The resulting time-of-flight spectrum was calibrated into a true mass spectrum using the H^+ and NO^+ ions at m/z 1 and 30, respectively. Mass spectra were generally accumulated for $1.0x10^6$ fission events corresponding to 15-20 minutes. Resulting assigned masses all correspond to isotopically averaged molecular masses. The accuracy of mass assignment is generally better than 0.1 %.

MALDI-MS

MALDI-TOF MS analysis was carried out using a Voyager RP instrument (PerSeptive Biosystems Inc., Framingham, MA) equipped with delayed extraction and operated in linear mode. Alpha-cyano-4-hydroxy-cinnamic acid was used as matrix, and mass assignments were based on external calibration.

Synthetic methods

The peptide was synthesized on Fmoc protected Rink amide resin (Novabiochem) or chlorotrityl resin using Fmoc strategy on an Applied Biosystems 433A peptide synthesizer in 0.25 mmol scale using the manufacturer supplied FastMoc UV protocols which employ HBTU (2-(1H-Benzotriazol-1-yl-)-1,1,3,3 tetramethyluronium hexafluorophosphate) mediated couplings in NMP (N-methyl pyrrolidone) and UV monitoring of the deprotection of the Fmoc protection group. The protected amino acid derivatives used were standard Fmoc-amino acids (Anaspec) supplied in preweighed cartridges suitable for the ABI433A synthesizer with the exception of unnatural aminoacids such as Fmoc-Aib-OH (Fmoc-aminoisobutyric acid).

The attachment of sidechains and linkers to specific lysine residues on the crude resin bound protected peptide was carried out in a specific position by incorporation of Fmoc-Lys(Dde)-OH during automated synthesis.

<u>Procedure for removal of Fmoc-protection</u>:

<u>Procedure for attachment of sidechains to Lysine residues</u>

<u>Procedure for removal of Dde-protection</u>. Afterward the resin was placed in a manual shaker/filtration apparatus and treated with 2% hydrazine in DMF to remove the DDE group. Then sidechains were attached manually using HOBt/EDAC couplings in NMP and standard Fmoc-deprotection using 20% piperidine in DMF.

<u>Procedure for cleaving of peptide of resin</u>:

The peptide was cleaved from the resin by stirring for 240 min at room temperature with a mixture of triflouroacetic acid, water and trilsopropylsilane (95:2.5:2.5). The cleavage mixture was filtered and the filtrate was concentrated to an oil by a stream of nitrogen. The crude

peptide was precipitated from this oil with 45 ml diethyl ether and washed 3 times with 45 ml diethyl ether.

Purification:

The crude peptide was purified by semipreparative HPLC on a 20 mm x 250 mm column packed with 7□ C-18 silica. The column was equilibrated with 21% CH_3CN in 0.05M $(NH_4)_2SO_4$, which was adjusted to pH 2.5 with concentrated H_2SO_4. After drying the crude peptide was dissolved in 5 ml 70% CH_3CN / 0.1% TFA in H_2O and diluted to 50 ml with H_2O. 20 ml of this solution was diluted to 90 ml and injected on the column which then was eluted with a gradient of 21% - 31% CH_3CN in 0.05M $(NH_4)_2SO_4$, pH 2.5 at 10 ml/min during 47 min at 40 °C. The peptide containing fractions were collected and diluted with 3 volumes of H_2O and passed through a Sep-Pak® C18 cartridge (Waters part. #:51910) which has been equilibrated with 0.1% TFA. It was then eluted with 70% CH_3CN containing 0.1% TFA and the purified peptide was isolated by lyophilisation after dilution of the eluate with water

The final product obtained was characterised by analytical RP-HPLC (retention time) and by LCMS

The RP-HPLC analysis was performed using UV detection at 214 nm and a Vydac 218TP54 4.6mm x 250mm 5□ C-18 silica column (The Separations Group, Hesperia, USA) which was eluted at 1 ml/min at 42 °C. Two different elution conditions were used:

A1: Equilibration of the column with 5% CH_3CN in a buffer consisting of 0.1M $(NH_4)_2SO_4$, which was adjusted to pH 2.5 with concentrated H_2SO_4 and elution by a gradient of 5% to 60% CH_3CN in the same buffer during 50 min.

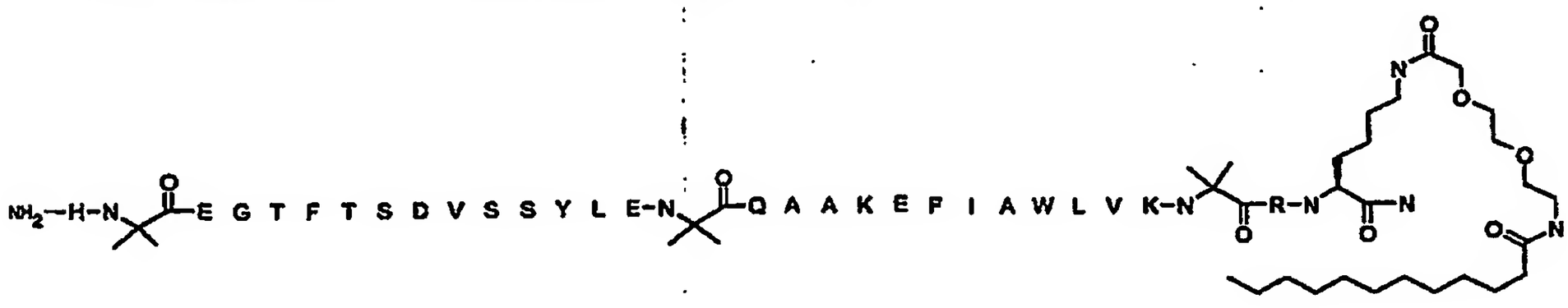
B1: Equilibration of the column with 5% CH_3CN / 0.1% TFA / H_2O and elution by a gradient of 5% CH_3CN / 0.1% TFA / H_2O to 60% CH_3CN / 0.1% TFA / H_2O during 50 min.

B6:

LCMS :

Example 1

$N^{\varepsilon 37}$-(2-(2-(2-(dodecylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$] GLP-1 H-(7-37)-NH_2



Prepared as in "Synthetic methods".

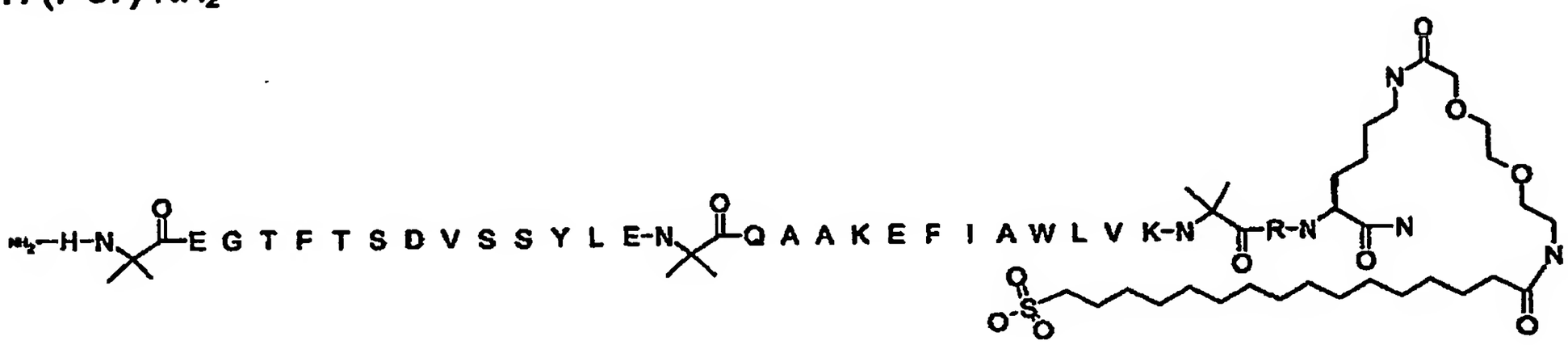
HPLC: (method B6): RT=32.8 min (98%)

HPLC: (method A1): RT=43.6 min

LCMS: m/z = 765.0 $(M+H)^{5+}$, 957.0 $(M+H)^{4+}$, 1275.0 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 3825.0

Example 2

$N^{\varepsilon 37}$-(2-(2-(2-(17-sulphohexadecanoylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,Lys37] GLP-1 H-(7-37)-NH_2



Prepared as in "Synthetic methods".

HPLC: (method A1): RT=45.5 min

LCMS: m/z = 792.9 $(M+H)^{5+}$, 990.9 $(M+H)^{4+}$, 1320.9 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 3959.9

Example 3

$N^{\varepsilon 37}$-{2-[2-(2-(15-carboxyhexadecanoylamino)ethoxy)ethoxy]acetyl}-[$Aib^{8,22,35}$,Lys^{37}] GLP-1 H-(7-37)-NH_2

Prepared as in "Synthetic methods".

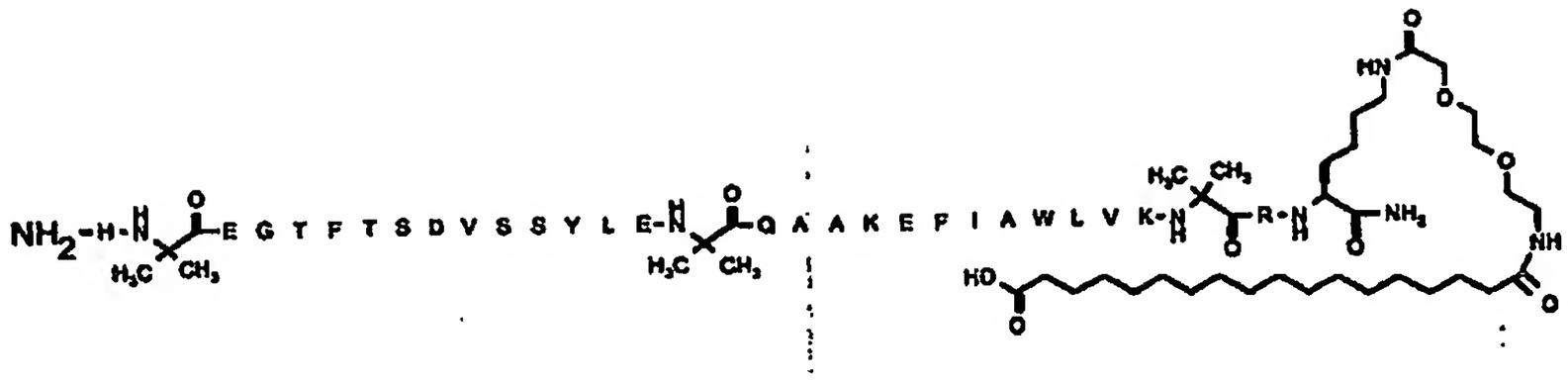
HPLC: (method B1): RT=43.8 min (92%)

HPLC: (method A1): RT=42.0 min

LCMS: m/z = 978.3 $(M+H)^{4+}$, 1303.8 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 3909.6

Example 4

$N^{\varepsilon 37}$-(2-(2-(2-(17-carboxyheptadecanoylamino)ethoxy)ethoxy)acetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1 H (7-37) -NH_2



Prepared as in "Synthetic methods".

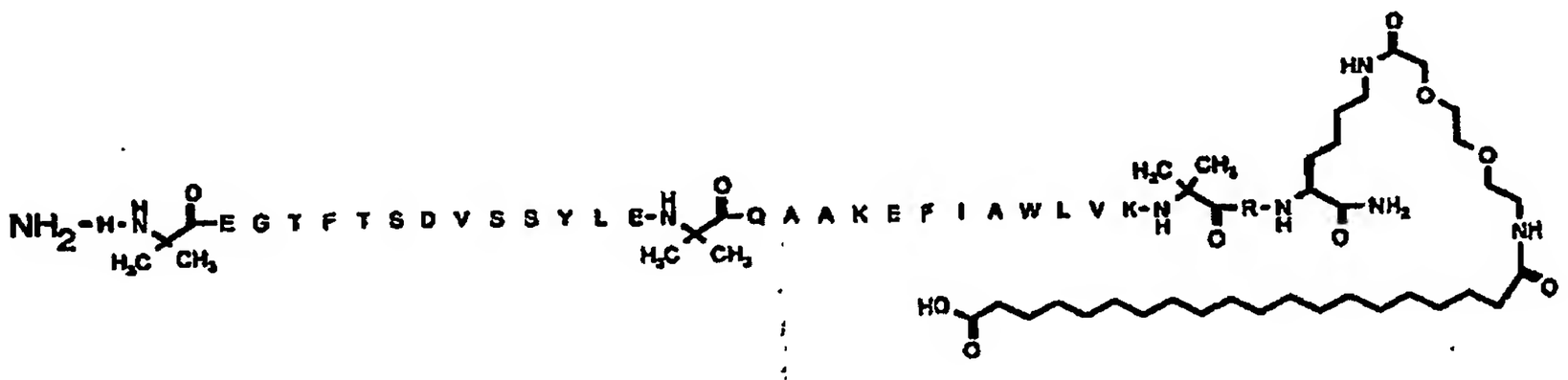
HPLC: (method B1): RT=46.4 min (100%)

HPLC: (method A1): RT=44.4 min

LCMS: m/z = 985.5 $(M+H)^{4+}$, 1313.4 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 3937.6

Example 5

$N^{\varepsilon 37}$-(2-(2-(2-(19-carboxynonadecanoylamino)ethoxy)ethoxy)acetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1 H (7-37) -NH_2



Prepared as in "Synthetic methods".

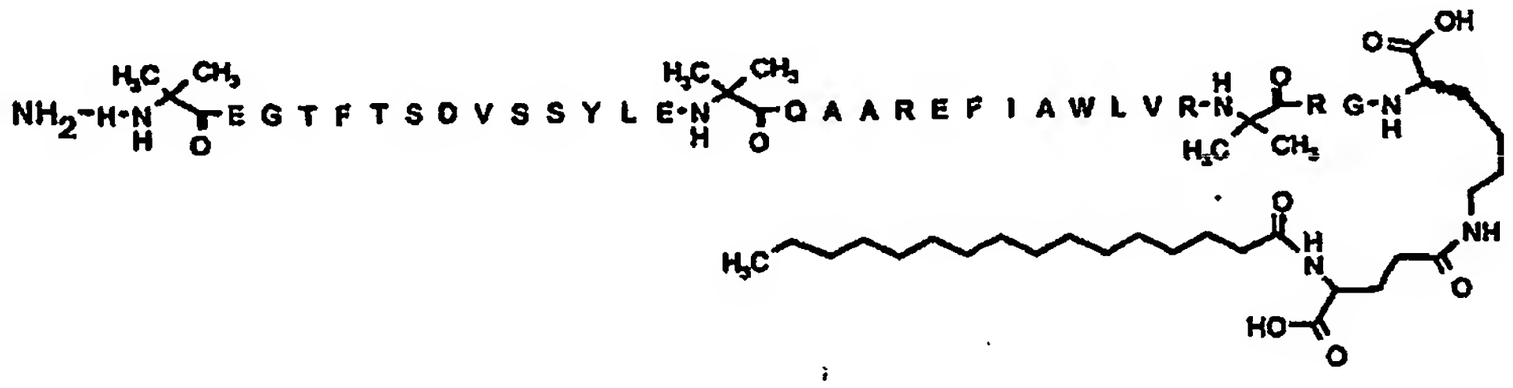
HPLC: (method B1): RT=49.5 min (100%)

HPLC: (method A1): RT=47.1 min

LCMS: m/z = 992.5 $(M+H)^{4+}$, 1322.6 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 3965.7

Example 6

$N^{\varepsilon 38}$-(4-(hexadecanoylamino)-4(S)-carboxybutyryl)-[$Aib^{8,22,35}$,$Arg^{26,34}$] GLP-1 H-(7-37) Lys NH_2



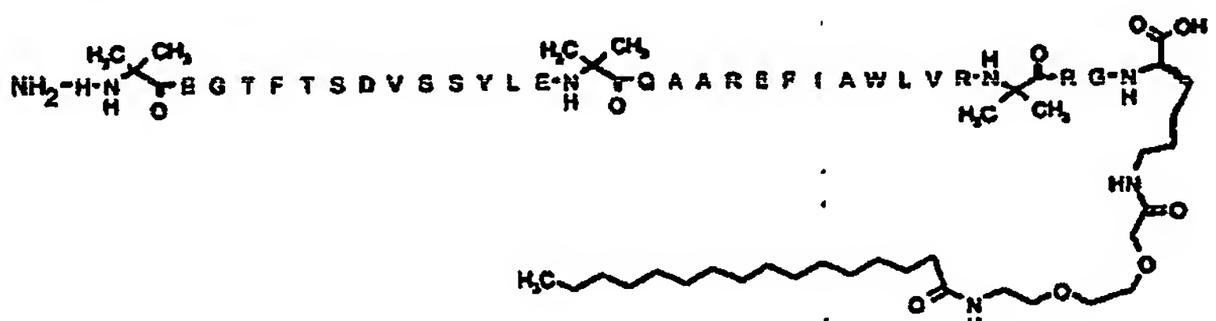
Prepared as in "Synthetic methods".

HPLC: (method B6): RT=36.28min 49.5 min (100%)

LCMS: m/z = 995 $(M+H)^{4+}$, 1326 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 3977.6

Example 7

$N^{\epsilon 38}$-(2-(2-(2-(hexadecanoylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,$Arg^{26,34}$] GLP-1 H-(7-37)Lys NH_2



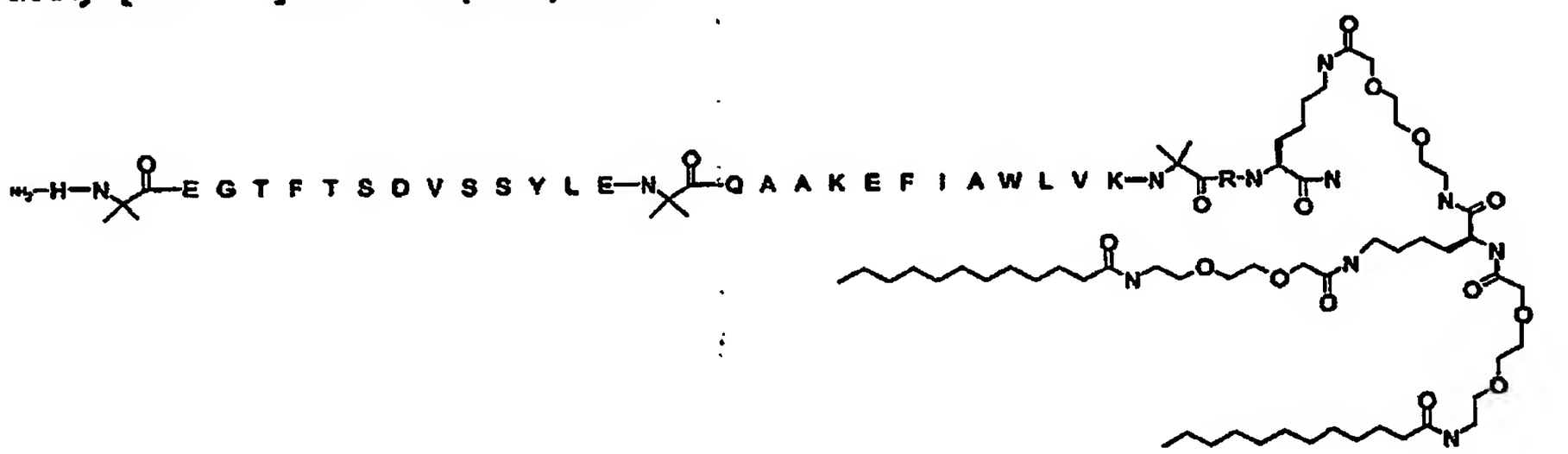
Prepared as in "Synthetic methods".

HPLC: (method B6): RT=37.1min (100%)

LCMS: m/z = 999 $(M+H)^{4+}$, 1332 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 3993.7

Example 8

$N^{\epsilon 37}$(2-[2-(2,6-(S)-Bis-{2-[2-(2-(dodecanoylamino)ethoxy)ethoxy]acetylamino}hexanoylamino)ethoxy]ethoxy}) acetyl-[$Aib^{8,22,35}$]GLP-1 H-(7-37)-NH_2



Prepared as in "Synthetic methods".

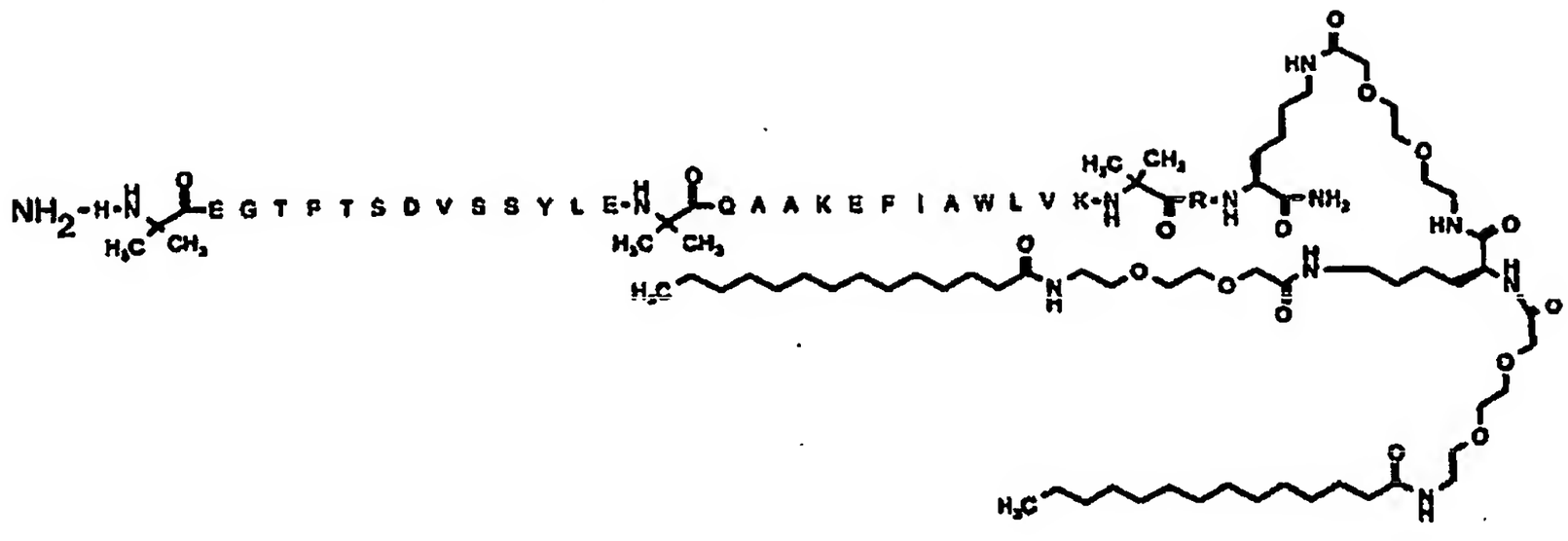
HPLC: (method B6): RT=38.2 min (100%)

LCMS: m/z = 1106.7 $(M+H)^{4+}$, 1475.3 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4433.0

Example 9

$N^{\epsilon 37}$-(2-[2-(2,6-(S)-Bis-{2-[2-(2-(tetradecanoylamino)ethoxy)ethoxy]acetylamino}hexanoylamino)ethoxy]ethoxy})

acetyl-[Aib^{8,22,35}]GLP-1 H-(7-37)-NH_2



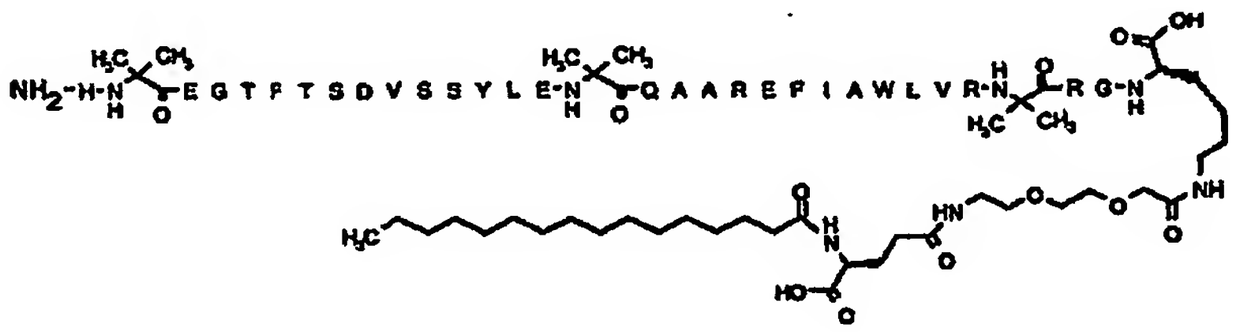
Prepared as in "Synthetic methods".

HPLC: (method B6): RT=42.9 min (90%)

LCMS: m/z = 1120.9 $(M+H)^{4+}$, 1494.2 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4480.4

Example 10

$N^{\varepsilon 38}$-epsilon38-(2-(2-(2-(4-(hexadecanoylamino)-4(S)-carboxybutyrylamino)ethoxy)ethoxy)acetyl)-[Aib^{8,22,35},Arg^{26,34}] GLP-1 H-(7-37) Lys-OH



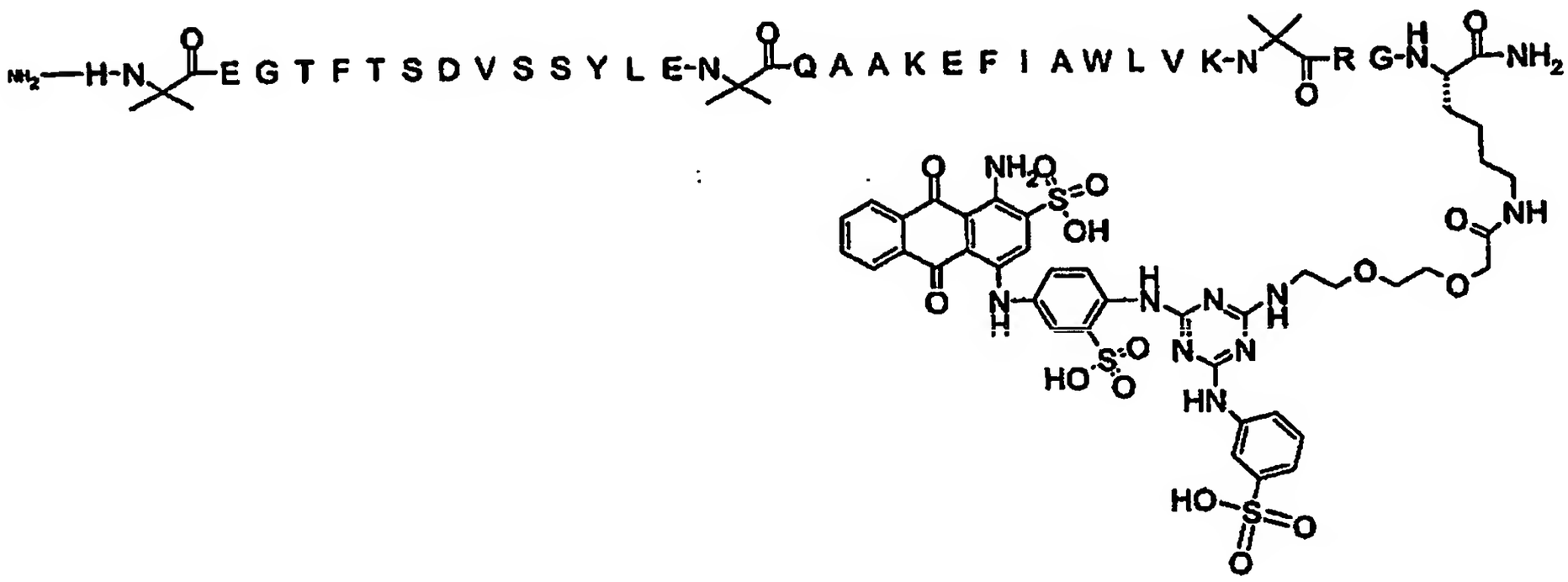
Prepared as in "Synthetic methods".

HPLC: (method B6): RT=36.0 min (100%)

LCMS: m/z = 1032.0 $(M+H)^{4+}$, 1374.0 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4122.8

Example 11

$N^{\varepsilon 38}$-(2-{2-[4-[4-(4-Amino-9,10-dioxo-3-sulfo-9,10-dihydro-anthracen-1-ylamino)-2-sulfo-phenylamino]-6-(2-sulfo-phenylamino)-[1,3,5]triazin-2-ylamino]-ethoxy}-ethoxy)-acetyl)-[Aib^{8,22,35}] GLP-1 H (7-37)Lys-NH_2



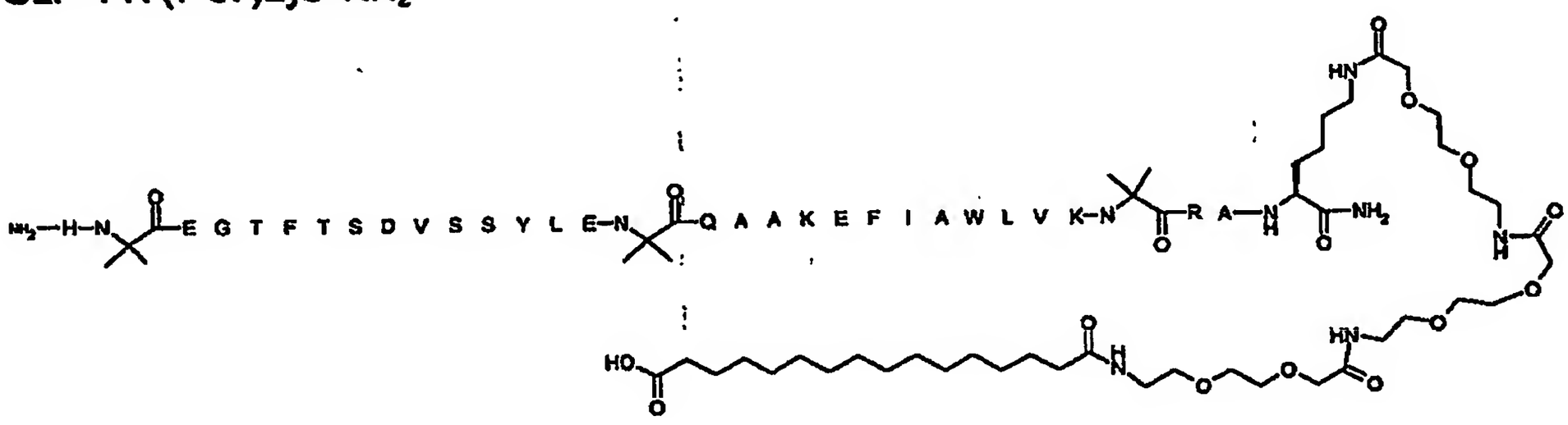
Prepared by loading DdeLys(Fmoc)-OH onto Rink resin. The resin was then treated with piperidine as in "Synthetic methods" to remove Fmoc selectively. 2-(2-(2-(Fmoc-amino)ethoxy)ethoxy)acetic acid was coupled onto the epsilon amingroup of lysine and Fmoc was removed. DMSO and Cibacron Blue 3GA (17 equivalents) (Sigma C-9534) was added and the mixture was heated at 60 °C for 15 hours, washed with water (3 times), methanol (2 times), THF (2 times) and diethyl ether (2 times). The Dde protecting group was removed and the remaining amino acids were added as in "Synthetic methods"

HPLC: (method A1): RT=38.1 min (92%)

LCMS: m/z = 1110.4 $(M+H)^{4+}$, 1436.4 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4435.9

Example 12

$N^{\epsilon 38}$-({2-[2-(2-{2-[2-(2-{2-[2-(15-carboxypentadecanoylamino)-ethoxy]ethoxy}acetylamino)ethoxy]ethoxy}acetyl amino)ethoxy]ethoxy}acetyl)- [$Aib^{8,22,35}$] GLP-1 H (7-37)Lys- NH_2



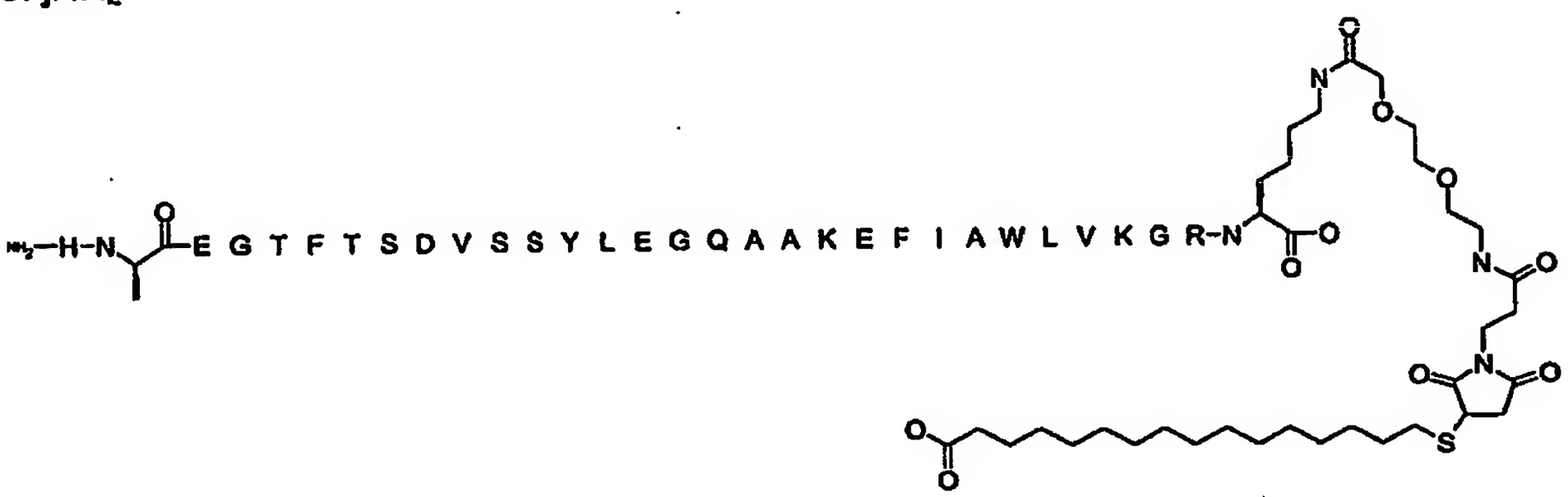
HPLC: (method A1): RT=41.2 min (93%)

HPLC: (method B6): RT=30.7 min

LCMS: m/z = 1069.1 $(M+H)^{4+}$, 1424.6 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4271

Example 13

$N^{\epsilon 38}$-([2-(2-{3-[2,5-dioxo-3-(15-carboxypentadecylsulfanyl)-pyrrolidin-1-yl]-propionylamino}ethoxy)ethoxy)acetyl]-[D-Ala^8,Lys^{37}]-GLP-1-[7-37]NH_2

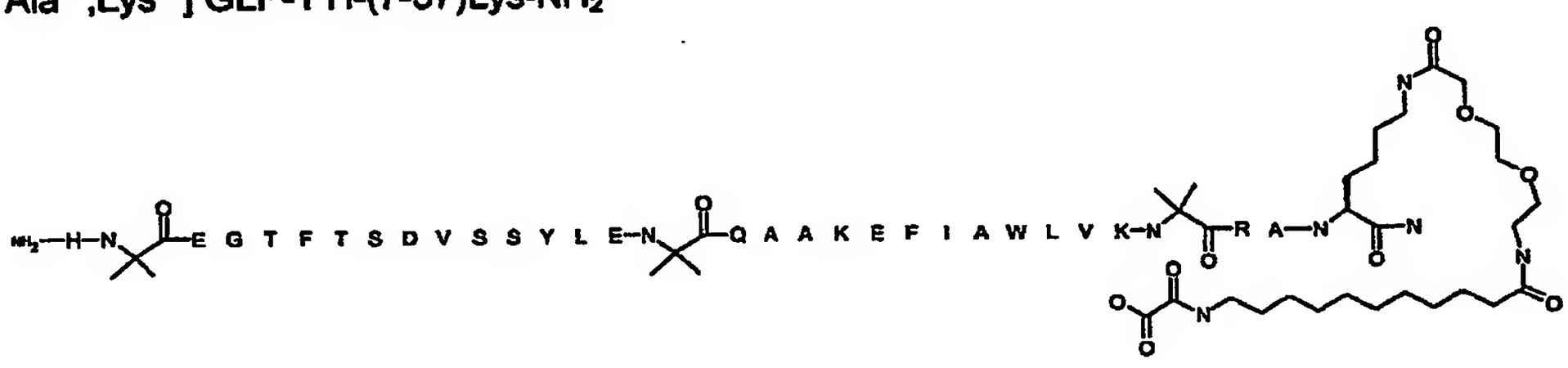


HPLC: (method A1): RT=45.2 min (100%)

LCMS: m/z = 1004.0 $(M+H)^{4+}$, 1338.2 $(M+H)^{3+}$ Calculated $(M+H)^+$ = 4010.7

Example 14

$N^{\epsilon 38}$-(2-(2-(2-(11-(oxalylamino)undecanoylamino)ethoxy)ethoxy)acetyl-)) [Aib^{8,22,35}, Ala^{37},Lys^{38}] GLP-1 H-(7-37)Lys-NH_2



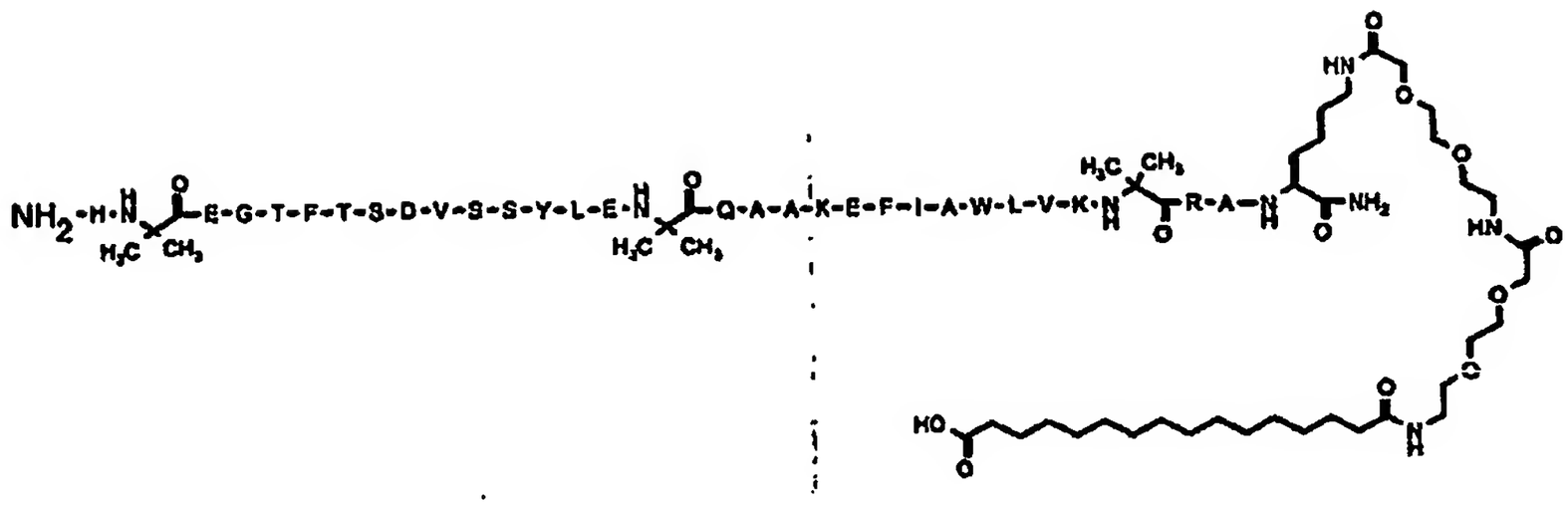
HPLC (method A1): RT=37.9 min (100%)

HPLC (method B1): RT=39.5 min (100%)

LCMS: m/z = 993.3 $(M+H)^{4+}$, 1323.9 $(M+H)^{3+}$ Calculated $(M+H)^+$ = 3967.6

Example 15

$N^{\epsilon 38}$({2-[2-(2-{2-[2-(2-(15-carboxy-pentadecanoylamino)-ethoxy]ethoxy}acetylamino)ethoxy]ethoxy}acetyl)[Aib^{8,22,35},Ala^{37}]-GLP-1[7-37]Lys NH_2



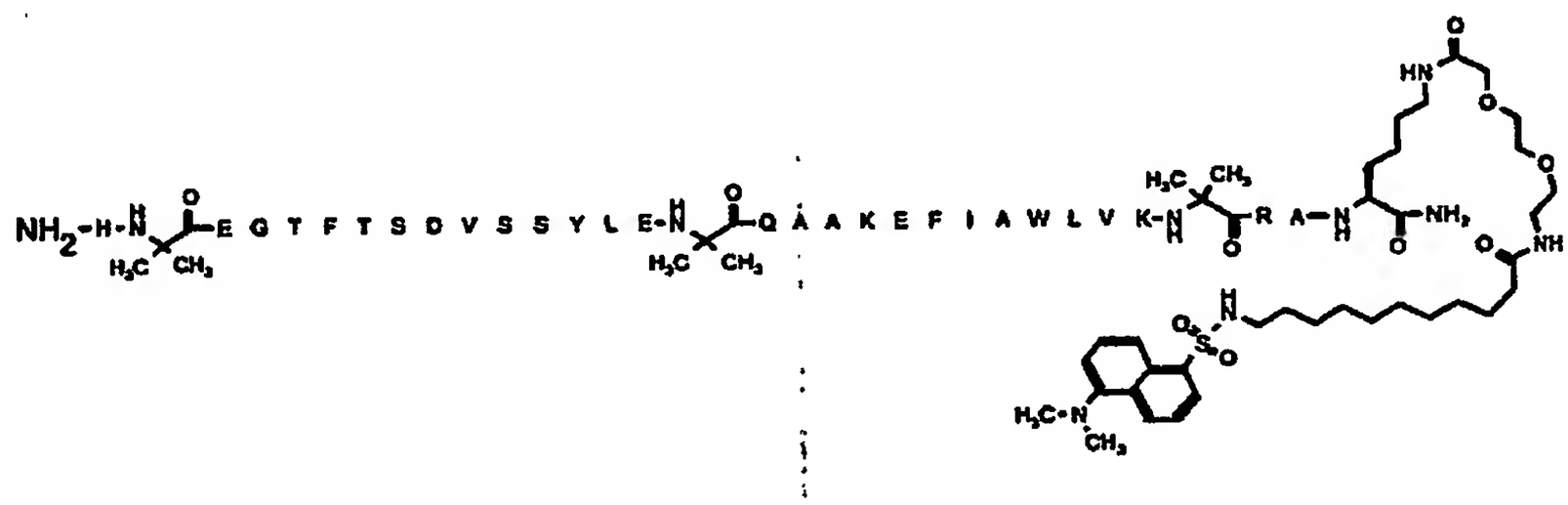
HPLC (method B6): RT=31.1 min (94%)

HPLC (method A1): RT=41.9 min

LCMS: m/z = 1376.3 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4125.8

Example 16

$N^{\varepsilon 38}$-((2-{2-[11-(5-Dimethylaminonaphthalene-1-sulfonylamino)undecanoylamino]ethoxy}ethoxy)acetyl [$Aib^{8,22,35}$,Ala^{37}]-GLP-1[7-37]LysNH_2



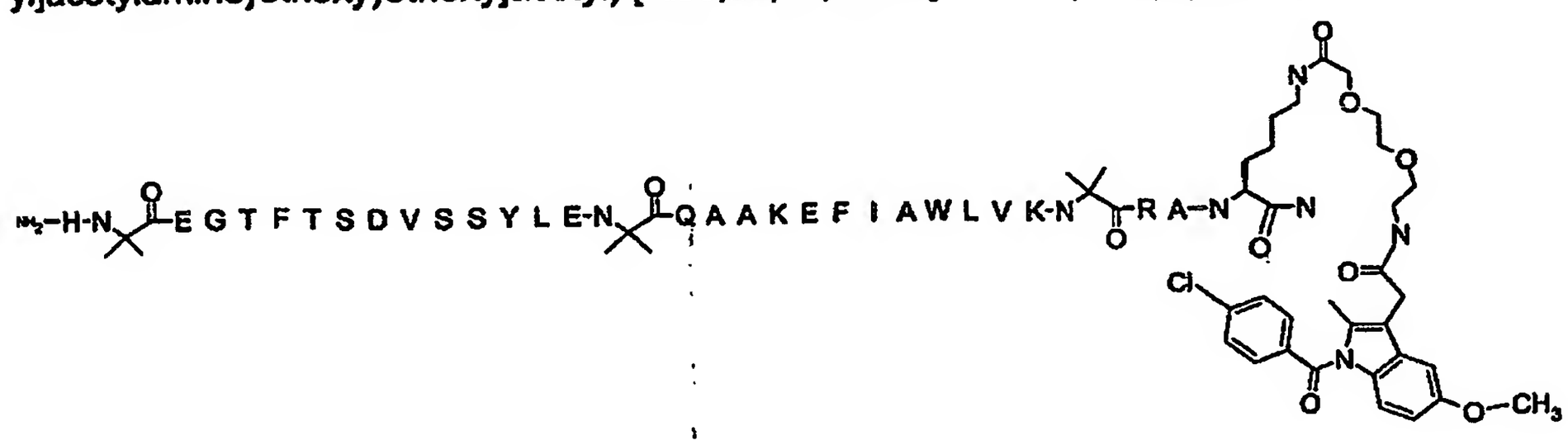
HPLC (method A1): RT=42.6 min (100%)

HPLC (method B6): RT=30.4 min

LCMS: m/z = 1377.3 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4128.8

Example 17

$N^{\varepsilon 38}$([2-(2-{2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetylamino}ethoxy)ethoxy]acetyl) [Aib8,22,35,Ala37]-GLP-1(7-37)Lys-NH_2



HPLC (method A1): RT=41.1 min (98%)

HPLC (method B6): RT=31.1 min

LCMS: m/z = 1351.8 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4052.0

Example 18

$N^{\varepsilon 38}$-2-(2-(2-(octadecanoylamino)ethoxy)ethoxy)acetyl [Aib^{8},$Arg^{26,34}$,$Glu^{22,23,30}$]GLP-1 H(7-37)Lys-NH_2

HPLC (method B6): RT=39.3 min (89.9%)

LCMS: m/z = 1366.6 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4095.6

Example 19

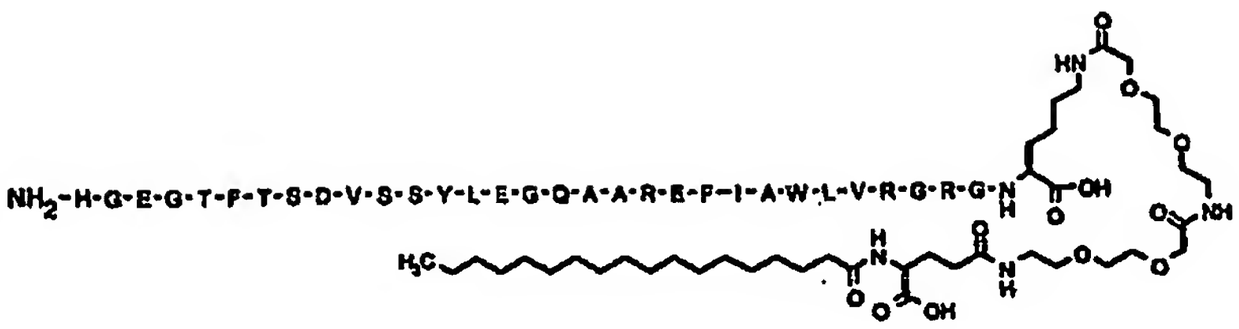
$N^{\varepsilon 38}$-2-(2-(2-(eicosanoylamino)ethoxy)ethoxy)acetyl [Aib^{8},$Arg^{26,34}$,$Glu^{22,23,30}$]GLP-1 H(7-37)Lys-NH_2

HPLC (method B6): RT=42.6 min (88.5%)

LCMS: m/z = 1375.7 $(M+H)^{3+}$ Calculated $(M+H)^{+}$ = 4123.7

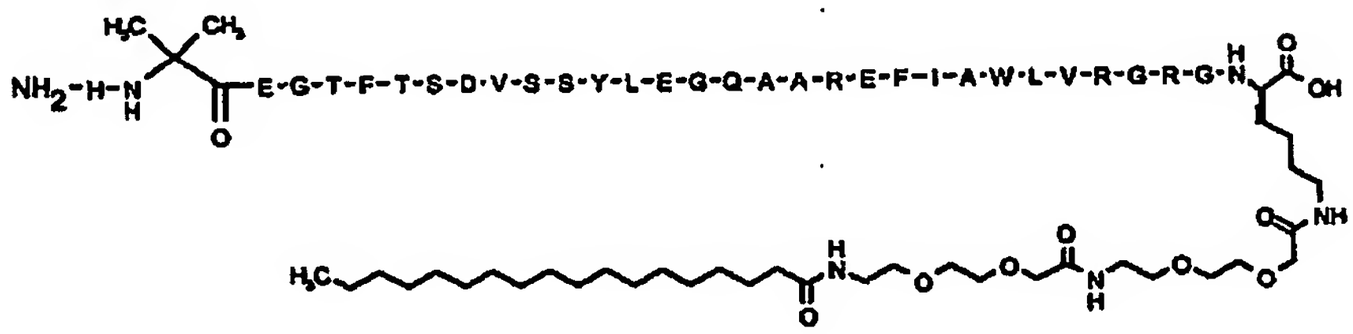
Example 20

Gly8,Arg26,34,36,Lys38 (N-epsilon({2-[2-(2-{2-[2-(4-Carboxy-4-octadecanoylamino-butyrylamino)ethoxy]ethoxy}acetylamino)ethoxy]ehoxy}-acetic acid))GLP-1 (7-38)



HPLC MS (incl methode): Rf = , mw =

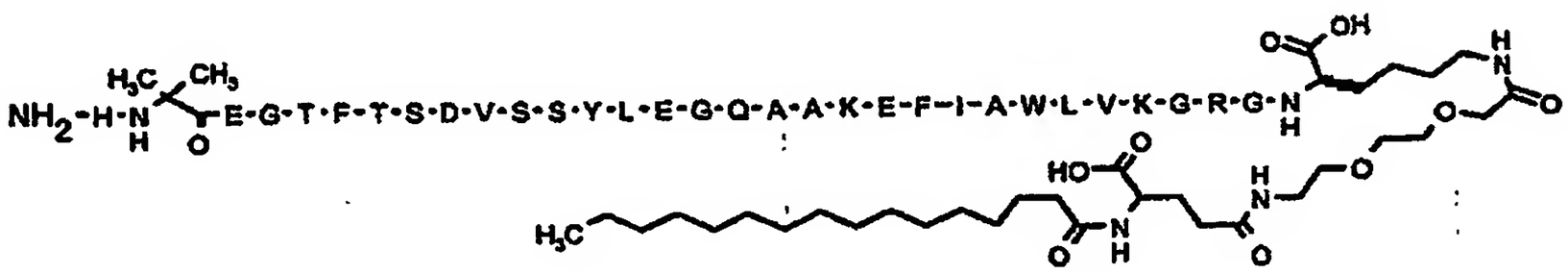
Example 21



HPLC MS (incl methode): Rf = , mw =

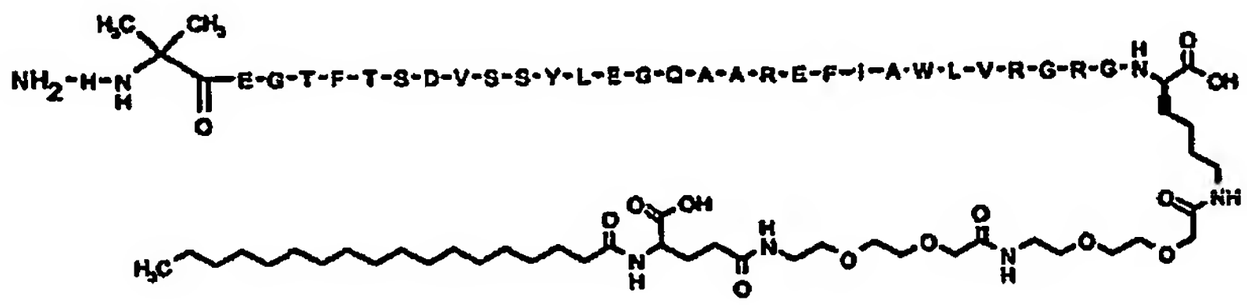
Example 22

Alb8,Lys38-(N-epsilon-2-(2-(2-(gamma-glutamyl(N-alfa-hexadecanoyl))aminoethoxy)ethoxy)acetyl)-GLP-1 (7-38)



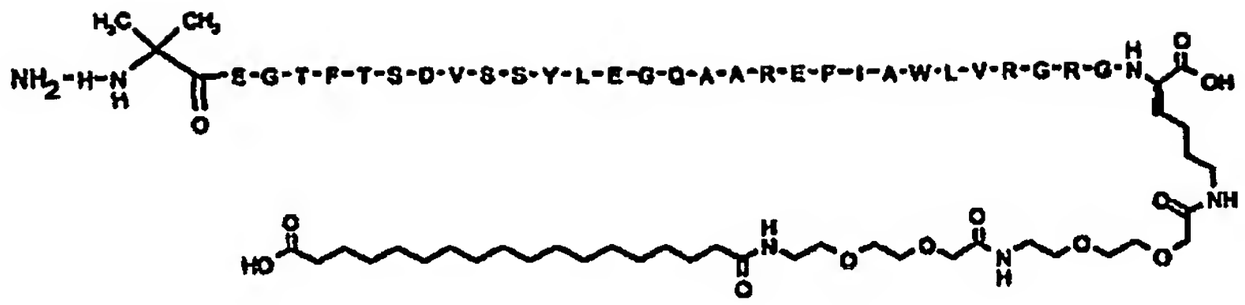
HPLC MS (incl methode): Rf = , mw =

Example 23



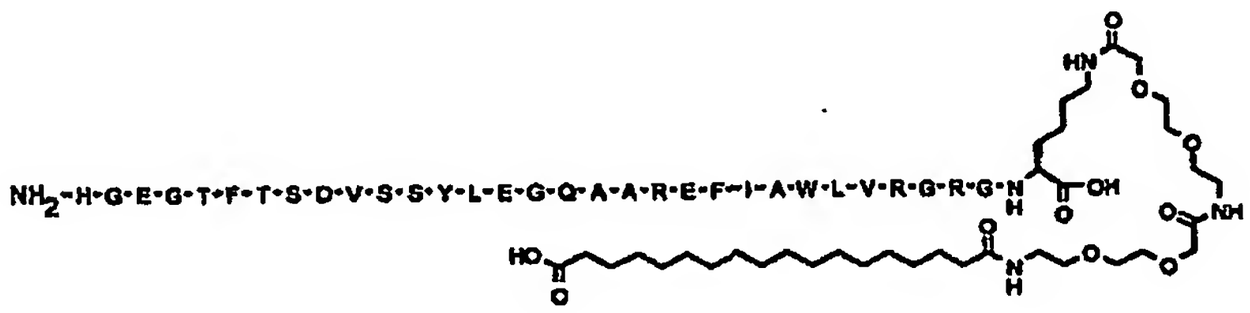
HPLC MS (incl methode): Rf = , mw =

Example 24



HPLC MS (incl methode): Rf = , mw =

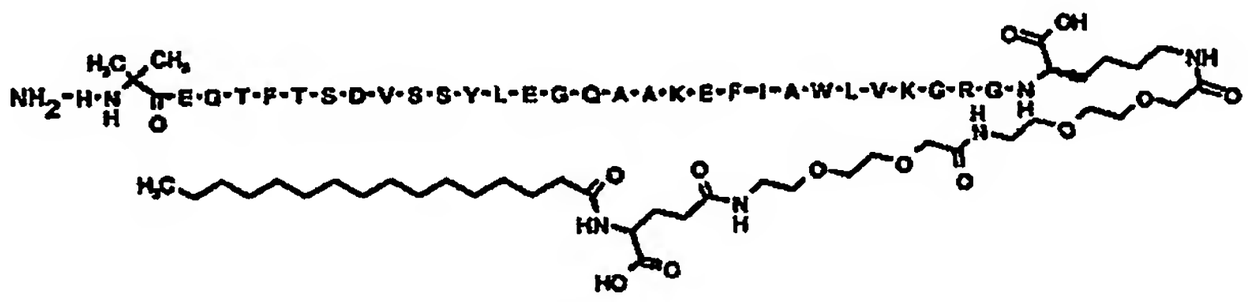
Example 25



HPLC MS (incl methode): Rf = , mw =

Example 26

Aib8,Lys38-(N-epsilon-2-(2-(2-(2-(2-(2-(gamma-glutamyl(N-alfa-hexadecanoyl))aminoethoxy)ethoxy)acetyl) aminoethoxy)ethoxy)acetyl-GLP-1 (7-38)



HPLC MS (incl methode): Rf = , mw =

Example 27

HPLC MS (incl methode): Rf = , mw =

Example 28

HPLC MS (incl methode): Rf = , mw =

Example 29

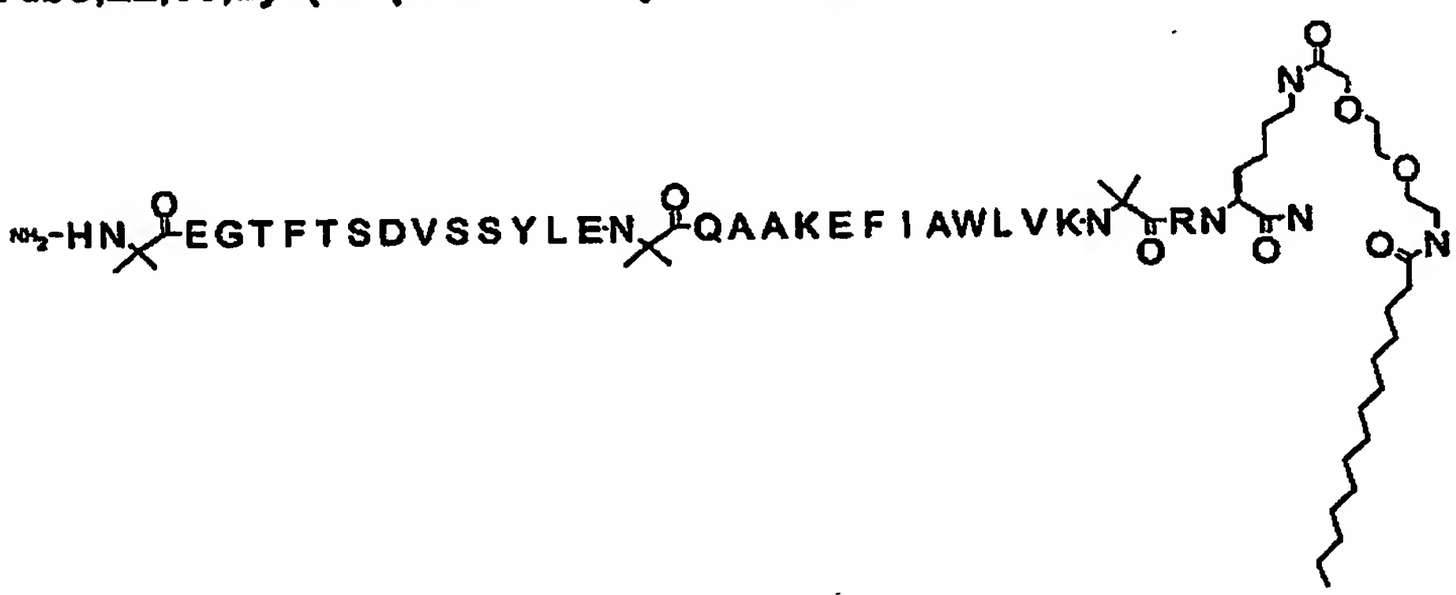
[Aib8,22,35,Lys(C12oyl-Aminoethoxy-ethoxyAcetyl-)37] GLP-1 H-(7-37)-NH2

HPLC: (method B6): RT=32,8 min (98%)

LC-MS: m/z = 765,7 (M+H)5+, 957,0 (M+H)4+, 1275,7 (M+H3+) = 3822,9

Example 30

Aib8,22,35,Lys(N-epsilon-C14oyl-aminoethoxyethoxyacetyl-)37] GLP-1 -(7-37)-NH2

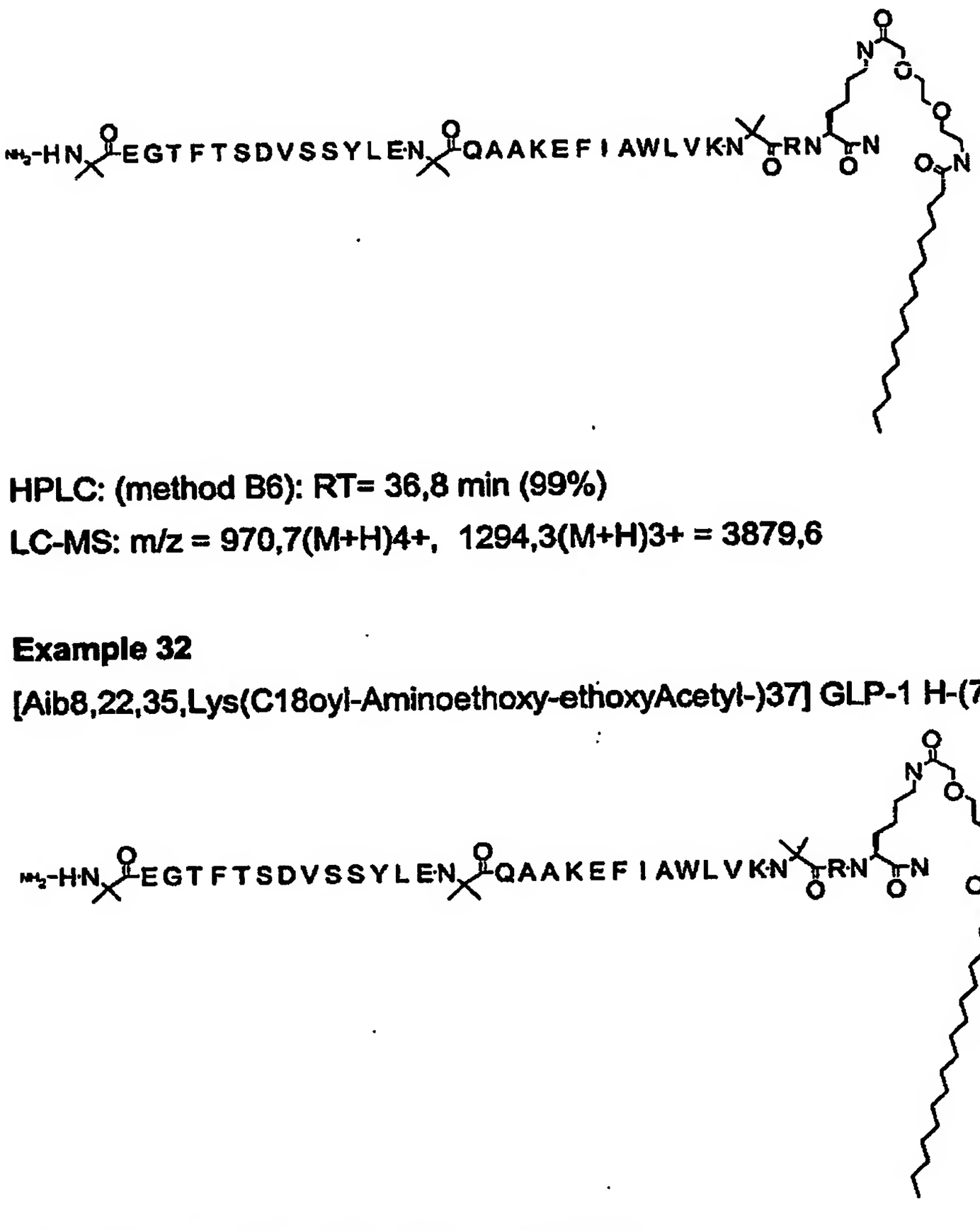


HPLC: (method B6): RT= 34,6 min (96%)

LC-MS: m/z = 771,4 (M+H)5+, 964,1(M+H)4+, 1284,9(M+H3+) = 3851,5

Example 31

[Aib8,22,35,Lys(C16oyl-Aminoethoxy-ethoxyAcetyl-)37] GLP-1 H-(7-37)-NH2



HPLC: (method B6): RT= 36,8 min (99%)

LC-MS: m/z = 970,7(M+H)4+, 1294,3(M+H)3+ = 3879,6

Example 32

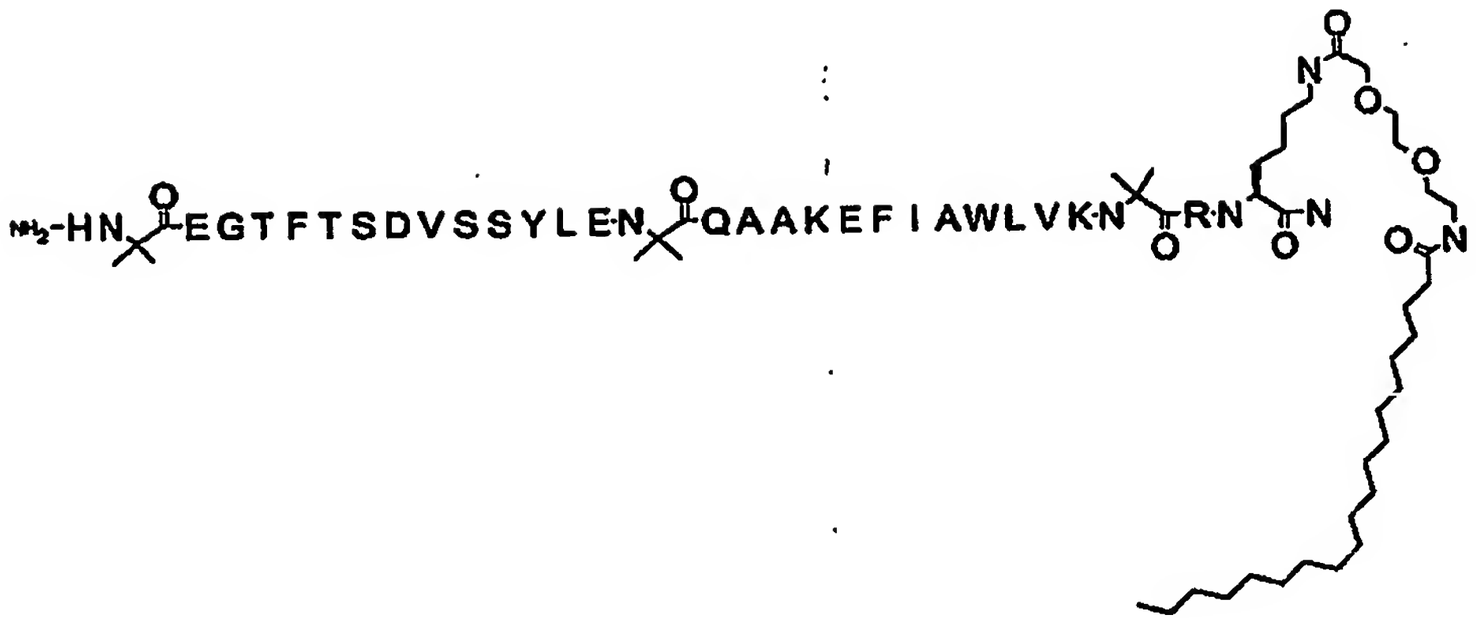
[Aib8,22,35,Lys(C18oyl-Aminoethoxy-ethoxyAcetyl-)37] GLP-1 H-(7-37)-NH2

HPLC: (method B6): RT= 39,4 min (100%)

LC-MS: m/z = 977,9 (M+H)4+, 1303,7(M+H3+) = 3907,6

Example 33

[Aib8,22,35,Lys(C20oyl-Aminoethoxy-ethoxyAcetyl-)37] GLP-1 H-(7-37)-NH2



HPLC: (method B6): RT= 42,7min (100%)

LC-MS: m/z = 984,8(M+H)4+, 1312,8(M+H3+) = 3935,7

BIOLOGICAL FINDINGS

Protraction of GLP-1 derivatives after s.c. administration

The protraction of a number GLP-1 derivatives of the invention was determined by monitoring the concentration thereof in plasma after sc administration to healthy pigs, using the method described below. For comparison also the concentration in plasma of GLP-1(7-37) after sc. administration was followed. The protraction of other GLP-1 derivatives of the invention can be determined in the same way.

Pigs (50% Duroc, 25% Yorkshire, 25% Danish Landrace, app 40 kg) were fasted from the beginning of the experiment. To each pig 0.5 nmol of test compound per kg body weight was administered in a 50 µM isotonic solution (5 mM phosphate, pH 7.4, 0.02% Tween®-20 (Merck), 45 mg/ml mannitol (pyrogen free, Novo Nordisk). Blood samples were drawn from a catheter in vena jugularis. 5 ml of the blood samples were poured into chilled glasses containing 175 µl of the following solution: 0.18 M EDTA, 1500 KIE/ml aprotinin (Novo Nordisk) and 3% bacitracin (Sigma), pH 7.4. Within 30 min, the samples were centrifuged for 10 min at 5-6000*g. Temperature was kept at 4°C. The supernatant was pipetted into different glasses and kept at minus 20°C until use.

The plasma concentrations of the peptides were determined by RIA using a monoclonal antibody specific for the N-terminal region of GLP-1(7-37). The cross reactivities were less than 1% with GLP-1(1-37) and GLP-1(8-36)amide and < 0.1% with GLP-1(9-37), GLP-1(10-36)amide and GLP-1(11-36)amide. The entire procedure was carried out at 4°C.

The assay was carried out as follows: 100 µl plasma was mixed with 271 µl 96% ethanol, mixed using a vortex mixer and centrifuged at 2600*g for 30 min. The supernatant was decanted into Minisorp tubes and evaporated completely (Savant Speedvac AS290). The evaporation residue was reconstituted in the assay buffer consisting of 80 mM NaH_2PO_4/Na_2HPO_4, 0.1 % HSA (Orpha 20/21, Behring), 10 mM EDTA, 0.6 mM thiomersal (Sigma), pH 7.5. Samples were reconstituted in volumes suitable for their expected concentrations, and were allowed to reconstitute for 30 min. To 300 µl sample, 100 µl antibody solution in dilution buffer containing 40 mM NaH_2PO_4/Na_2HPO_4, 0.1 % HSA, 0.6 mM thiomersal, pH 7.5, was added. A non-specific sample was prepared by mixing 300 µl buffer with 100 µl dilution buffer. Individual standards were prepared from freeze dried stocks, dissolved in 300 µl assay buffer. All samples were pre-incubated in Minisorp tubes with antibody as described above for 72 h. 200 µl tracer in dilution buffer containing 6-7000 CPM was added, samples were mixed and incubated for 48 h. 1.5 ml of a suspension of 200 ml per litre of heparin-stabilised bovine plasma and 18 g per litre of activated carbon (Merck) in 40 mM NaH_2PO_4/Na_2HPO_4, 0.6 mM thiomersal, pH 7.5, was added to each tube. Before use, the suspension was mixed and allowed to stand for 2 h at 4°C. All samples were incubated for 1 h at 4°C and then centrifuged at 3400*g for 25 min. Immediately after the centrifugation, the supernatant was decanted and counted in a γ-counter. The concentration in the samples was calculated from individual standard curves. Plasma concentrations were found, calculated as % of the maximum concentration for the individual compounds (n=2). The GLP-1 derivatives of the invention have a protracted profile of action relative to GLP-1(7-37) and are much more persistent in plasma than GLP-1(7-37). The time at which the peak concentration in plasma is achieved varies within wide limits, depending on the particular GLP-1 derivative selected.

Stimulation of cAMP formation in a cell line expressing the cloned human GLP-1 receptor

In order to demonstrate efficacy of the GLP-1 derivatives, their ability to stimulate formation of cAMP in a cell line expressing the cloned human GLP-1 receptor was tested. An EC_{50} was calculated from the dose-response curve.

Baby hamster kidney (BHK) cells expressing the human pancreatic GLP-1 receptor were used (Knudsen and Pridal, 1996, Eur. J. Pharm. 318, 429-435). Plasma membranes were prepared (Adelhorst *et al*, 1994, J. Biol. Chem. 269, 6275) by homogenisation in buffer (10 mmol/l Tris-

HCl and 30 mmol/l NaCl pH 7.4, containing, in addition, 1 mmol/l dithiothreitol, 5 mg/l leupeptin (Sigma, St. Louis, MO, USA), 5 mg/l pepstatin (Sigma, St. Louis, MO, USA), 100 mg/l bacitracin (Sigma, St. Louis, MO, USA), and 16 mg/l aprotinin (Novo Nordisk A/S, Bagsvaerd, Denmark)). The homogenate was centrifuged on top of a layer of 41 w/v% sucrose. The white band between the two layers was diluted in buffer and centrifuged. Plasma membranes were stored at -80°C until used.

The assay was carried out in 96-well microtiter plates in a total volume of 140 µl. The buffer used was 50 mmol/l Tris-HCl, pH 7.4 with the addition of 1 mmol/l EGTA, 1.5 mmol/l $MgSO_4$, 1.7 mmol/l ATP, 20 mM GTP, 2 mmol/l 3-isobutyl-1-methylxanthine, 0.01 % Tween-20 and 0.1 % human serum albumin (Reinst, Behringwerke AG, Marburg, Germany). Compounds to be tested for agonist activity were dissolved and diluted in buffer, added to the membrane preparation and the mixture was incubated for 2 h at 37°C. The reaction was stopped by the addition of 25 µl of 0.05 mol/l HCl. Samples were diluted 10 fold before analysis for cAMP by a scintillation proximity assay (RPA 538, Amersham, UK).

CLAIMS

1. A compound which comprises a therapeutic polypeptide linked to an albumin binding residue via a hydrophilic spacer.

2. A compound which comprises a therapeutic polypeptide linked to an albumin binding residue via a hydrophilic spacer $-(CH_2)_lD[(CH_2)_nE]_m(CH_2)_pQ_q-$, wherein

l, m and n independently are 1-20 and p is 0-10,

Q is $-Z-(CH_2)_lD[(CH_2)_nG]_m(CH_2)_p-$,

q is an integer in the range from 0 to 5,

each D, E, and G independently are selected from -O-, $-NR^3-$, $-N(COR^4)-$, $-PR^5(O)-$, and $-P(OR^6)(O)-$, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from -C(O)NH-, $-C(O)NHCH_2-$, -OC(O)NH -, $-C(O)NHCH_2CH_2-$, $-C(O)CH_2-$, -C(O)CH=CH-, $-(CH_2)_s-$, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1

or a pharmaceutically acceptable salt or prodrug thereof.

3. A compound according to claim 2, which has the formula (I) :

A—W—B—Y—therapeutic polypeptide (I)

wherein

A is an albumin binding residue,

B is a hydrophilic spacer being $-(CH_2)_lD[(CH_2)_nE]_m(CH_2)_pQ_q-$, wherein

l, m and n independently are 1-20 and p is 0-10,

Q is $-Z-(CH_2)_lD[(CH_2)_nG]_m(CH_2)_p-$,

q is an integer in the range from 0 to 5,

each D, E, and G independently are selected from -O-, $-NR^3-$, $-N(COR^4)-$, $-PR^5(O)-$, and $-P(OR^6)(O)-$, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from -C(O)NH-, $-C(O)NHCH_2-$, -OC(O)NH -, $-C(O)NHCH_2CH_2-$, $-C(O)CH_2-$, -C(O)CH=CH-, $-(CH_2)_s-$, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1,

Y is a chemical group linking B and the therapeutic agent, and

W is a chemical group linking A and B.

4. A compound according to claim 2, which has the formula (II)

A–W—B—Y–therapeutic polypeptide —Y′–B′-W′—A′ (II)

wherein

A and A′ are albumin binding residues,

B and B′ are hydrophilic spacers independently selected from -$(CH_2)_l$D [$(CH_2)_n$E]$_m$($CH_2)_p$-Q_q-,

wherein

l, m and n independently are 1-20 and p is 0-10,

Q is -Z-$(CH_2)_l$D[$(CH_2)_n$G]$_m$($CH_2)_p$-,

q is an integer in the range from 0 to 5,

each D, E, and G independently are selected from -O-, -NR^3-, -N(COR^4)-, -PR^5(O)-, and -P(OR^6)(O)-, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from -C(O)NH-, -C(O)$NHCH_2$-, -OC(O)NH -, -C(O)$NHCH_2CH_2$-, -C(O)CH_2-, -C(O)CH=CH-, -$(CH_2)_s$-, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1,

Y is a chemical group linking B and the therapeutic agent, and

Y′ is a chemical group linking B′ and the therapeutic agent, and

W is a chemical group linking A and B, and

W′ is a chemical group linking A′ and B′.

5. A compound according to claim 4, wherein Y′ is selected from the group consisting of -C(O)NH-, -NHC(O)-, -C(O)$NHCH_2$-, -CH_2NHC(O)-, -OC(O)NH -, -NHC(O)O-, -C(O)$NHCH_2$-, CH_2NHC(O)-, -C(O)CH_2-, -CH_2C(O)-, -C(O)CH=CH-, -CH=CHC(O)-, -$(CH_2)_s$-, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and –C(O)NH-, wherein s is 0 or 1.

6. A compound according to any one of claims 4-5, wherein W′ is selected from the group consisting of -C(O)NH-, -NHC(O)-, -C(O)$NHCH_2$-, -CH_2NHC(O)-, -OC(O)NH -, -NHC(O)O-, -C(O)CH_2-, -CH_2C(O)-, -C(O)CH=CH-, -CH=CHC(O)-, -$(CH_2)_s$-, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and –C(O)NH-, wherein s is 0 or 1.

7. A compound according to claim 2, which has the formula (III)

A–W″-B—Y–therapeutic polypeptide (III)

|

A′

wherein

A and A´ are albumin binding residues,

B is a hydrophilic spacer selected from -$(CH_2)_lD[(CH_2)_nE]_m(CH_2)_p$-$Q_q$- wherein

l, m and n independently are 1-20 and p is 0-10,

Q is -Z-$(CH_2)_lD[(CH_2)_nG]_m(CH_2)_p$-,

q is an integer in the range from 0 to 5,

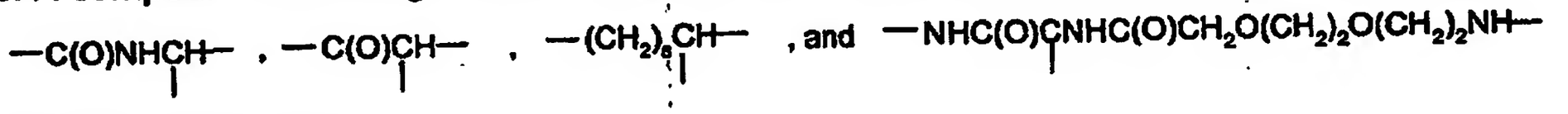
each D, E, and G are independently selected from -O-, -NR^3-, -$N(COR^4)$-, -$PR^5(O)$-, and -$P(OR^6)(O)$-, wherein R^3, R^4, R^5, and R^6 independently represent hydrogen or C_{1-6}-alkyl,

Z is selected from -C(O)NH-, -$C(O)NHCH_2$-, -OC(O)NH -, -$C(O)NHCH_2CH_2$-, -$C(O)CH_2$-, -C(O)CH=CH-, -$(CH_2)_s$-, -C(O)-, -C(O)O- or -NHC(O)-, wherein s is 0 or 1,

Y is a chemical group linking B and the therapeutic agent, and

W´´ is a chemical group linking B with A and A´.

8. A compound according to claim 7, wherein W´´ is selected from the group consisting of



, wherein s is 0, 1 or 2.

9. A compound according to any one of claims 3-8, wherein Y is selected from the group consisting of -C(O)NH-, -NHC(O)-, -$C(O)NHCH_2$-, -$CH_2NHC(O)$-, -OC(O)NH -, -NHC(O)O-, -$C(O)NHCH_2$-, $CH_2NHC(O)$-, -$C(O)CH_2$-, -$CH_2C(O)$-, -C(O)CH=CH-, -CH=CHC(O)-, -$(CH_2)_s$-, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and –C(O)NH-, wherein s is 0 or 1.

10. A compound according to any one of claims 3-9, wherein W is selected from the group consisting of -C(O)NH-, -NHC(O)-, -$C(O)NHCH_2$-, -$CH_2NHC(O)$-, -OC(O)NH -, -NHC(O)O-, -$C(O)CH_2$-, -$CH_2C(O)$-, -C(O)CH=CH-, -CH=CHC(O)-, -$(CH_2)_s$-, -C(O)-, -C(O)O-, -OC(O)-, -NHC(O)- and –C(O)NH-, wherein s is 0 or 1.

11. A compound according to any one of claims 2-10, wherein l is 1 or 2, n and m are independently 1-10 and p is 0-10.

12. A compound according to any one of claims 2-11, wherein D is –O-.

13. A compound according to any one of claims 2-12, wherein E is –O-.

14. A compound according to any one of claims 2-10, wherein the hydrophilic spacer is $-CH_2O[(CH_2)_2O]_m(CH_2)_pQ_q-$, where m is 1-10, p is 1-3, and Q is $-Z-CH_2O[(CH_2)_2O]_m(CH_2)_p-$.

15. A compound according to any of the previous claims, wherein q is 0 or 1.

16. A compound according to any of the previous claims, wherein q is 1.

17. A compound according to any one of claims 2-10 and 12-15, wherein G is –O-.

18. A compound according to any of the previous claims, wherein Z is selected from the group consisting of -C(O)NH-, $-C(O)NHCH_2-$, and –OC(O)NH-.

19. A compound according to any one of claims 2-15, wherein q is 0.

20. A compound according to any one of claims 2-13, wherein l is 2.

21. A compound according to any of the previous claims, wherein n is 2.

22. A compound according to any one of claims 2-15, wherein the hydrophilic spacer B is - $[CH_2CH_2O]_{m+1}(CH_2)_pQ_q-$.

23. A compound according to any of the previous claims, wherein the molar weight of said hydrophilic spacer is in the range from 80D to 1000D or in the range from 80D to 300D.

24. A compound according to any of the previous claims, wherein said albumin binding residue is a lipophilic residue.

25. A compound according to any of the previous claims, wherein said albumin binding residue binds non-covalently to albumin.

26. A compound according to any of the previous claims, wherein said albumin binding residue is negatively charged at physiological pH.

27. A compound according to any of the previous claims, wherein said albumin binding residue has a binding affinity towards human serum albumin that is below about 10 μM or below about 1 μM.

28. A compound according to any of the previous claims, wherein said albumin binding residue is selected from a straight chain alkyl group, a branched alkyl group, a group which has an ω-carboxylic acid group, a partially or completely hydrogenated cyclopentanophenanthrene skeleton.

29. A compound according to any of the previous claims, wherein said albumin binding residue is a cibacronyl residue.

30. A compound according to any of the previous claims, wherein said albumin binding residue has from 6 to 40 carbon atoms, from 8 to 26 carbon atoms or from 8 to 20 carbon atoms.

31. A compound according to any of the previous claims, wherein said albumin binding residue is a peptide, such as a peptide comprising less than 40 amino acid residues.

32. A compound according to any one of the previous claims, wherein the albumin binding residue via spacer and linkers is attached to said therapeutic polypeptide via the ϵ-amino group of a lysine residue.

33. A compound according to any one of the previous claims, wherein the albumin binding residue via spacer and linkers is attached to said therapeutic polypeptide via a linker to an amino acid residue selected from cysteine, glutamate and aspartate.

34. A compound according to any of the previous claims, wherein said therapeutic polypeptide is a GLP-1 peptide.

35. A compound according to claim 34, wherein said polypeptide is a GLP-1 peptide comprising the amino acid sequence of the formula (IV):

Xaa_7-Xaa_8-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Xaa_{16}-Ser-Xaa_{18}-Xaa_{19}-Xaa_{20}-Glu-Xaa_{22}-Xaa_{23}-Ala-Xaa_{25}-Xaa_{26}-Xaa_{27}-Phe-Ile-Xaa_{30}-Trp-Leu-Xaa_{33}-Xaa_{34}-Xaa_{35}-Xaa_{36}-Xaa_{37}-Xaa_{38}-Xaa_{39}-Xaa_{40}-Xaa_{41}-Xaa_{42}-Xaa_{43}-Xaa_{44}-Xaa_{45}-Xaa_{46}

Formula (IV) (SEQ ID No: 2)

wherein

Xaa_7 is L-histidine, D-histidine, desamino-histidine, 2-amino-histidine, β-hydroxy-histidine, homohistidine, N^{α}-acetyl-histidine, α-fluoromethyl-histidine, α-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine or 4-pyridylalanine;

Xaa_8 is Ala, Gly, Val, Leu, Ile, Lys, Aib, (1-aminocyclopropyl) carboxylic acid, (1-aminocyclobutyl) carboxylic acid, (1-aminocyclopentyl) carboxylic acid, (1-aminocyclohexyl) carboxylic acid, (1-aminocycloheptyl) carboxylic acid, or (1-aminocyclooctyl) carboxylic acid;

Xaa_{16} is Val or Leu;

Xaa_{18} is Ser, Lys or Arg;

Xaa_{19} is Tyr or Gln;

Xaa_{20} is Leu or Met;

Xaa_{22} is Gly, Glu or Aib;

Xaa_{23} is Gln, Glu, Lys or Arg;

Xaa_{25} is Ala or Val;

Xaa_{26} is Lys, Glu or Arg;

Xaa_{27} is Glu or Leu;

Xaa_{30} is Ala, Glu or Arg;

Xaa_{33} is Val or Lys;

Xaa_{34} is Lys, Glu, Asn or Arg;

Xaa_{35} is Gly or Aib;

Xaa_{36} is Arg, Gly or Lys;

Xaa_{37} is Gly, Ala, Glu, Pro, Lys, amide or is absent;

Xaa_{38} is Lys, Ser, amide or is absent.

Xaa_{39} is Ser, Lys, amide or is absent;

Xaa_{40} is Gly, amide or is absent;

Xaa_{41} is Ala, amide or is absent;

Xaa_{42} is Pro, amide or is absent;

Xaa_{43} is Pro, amide or is absent;

Xaa_{44} is Pro, amide or is absent;

Xaa_{45} is Ser, amide or is absent;

Xaa_{46} is amide or is absent ;

provided that if Xaa_{38}, Xaa_{39}, Xaa_{40}, Xaa_{41}, Xaa_{42}, Xaa_{43}, Xaa_{44}, Xaa_{45} or Xaa_{46} is absent then each amino acid residue downstream is also absent.

36. A compound according to claim 35, wherein said polypeptide is a GLP-1 peptide comprising the amino acid sequence of formula (V):

Xaa_7-Xaa_8-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Xaa_{18}-Tyr-Leu-Glu-Xaa_{22}-Xaa_{23}-Ala-Ala-Xaa_{26}-Glu-Phe-Ile-Xaa_{30}-Trp-Leu-Val-Xaa_{34}-Xaa_{35}-Xaa_{36}-Xaa_{37}-Xaa_{38}

Formula (V) (SEQ ID No: 3)

wherein

Xaa_7 is L-histidine, D-histidine, desamino-histidine, 2-amino-histidine, β-hydroxy-histidine, homohistidine, N^{α}-acetyl-histidine, α-fluoromethyl-histidine, α-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine or 4-pyridylalanine;

Xaa_8 is Ala, Gly, Val, Leu, Ile, Lys, Aib, (1-aminocyclopropyl) carboxylic acid, (1-aminocyclobutyl) carboxylic acid, (1-aminocyclopentyl) carboxylic acid, (1-aminocyclohexyl) carboxylic acid, (1-aminocycloheptyl) carboxylic acid, or (1-aminocyclooctyl) carboxylic acid;

Xaa_{16} is Ser, Lys or Arg;

Xaa_{22} is Gly, Glu or Aib;

Xaa_{23} is Gln, Glu, Lys or Arg;

Xaa_{26} is Lys, Glu or Arg;

Xaa_{30} is Ala, Glu or Arg;

Xaa_{34} is Lys, Glu or Arg;

Xaa_{35} is Gly or Aib;

Xaa_{36} is Arg or Lys;

Xaa_{37} is Gly, Ala, Glu or Lys;

Xaa_{38} is Lys, amide or is absent.

37. A compound according to any one of claims 34-36, wherein said GLP-1 peptide is selected from GLP-1(7-35), GLP-1(7-36), GLP-1(7-36)-amide, GLP-1(7-37), GLP-1(7-38), GLP-1(7-39), GLP-1(7-40), GLP-1(7-41) or an analogue thereof.

38. A compound according to any one of claims 34-37, wherein said GLP-1 peptide comprises no more than fifteen amino acid residues which have been exchanged, added or deleted as compared to GLP-1(7-37) (SEQ ID No. 1), or no more than ten amino acid residues which have been exchanged, added or deleted as compared to GLP-1(7-37) (SEQ ID No. 1).

39. A compound according to claim 38, wherein said GLP-1 peptide comprises no more than six amino acid residues which have been exchanged, added or deleted as compared to GLP-1(7-37) (SEQ ID No. 1).

40. A compound according to any one of claims 38-39, wherein said GLP-1 peptide comprises no more than 4 amino acid residues which are not encoded by the genetic code.

41. A compound according to claim 34, wherein said GLP-1 peptide is a DPPIV protected GLP-1 peptide.

42. A compound according to any one of claims 34-41, wherein said GLP-1 peptide comprises an Aib residue in position 8.

43. A compound according to any one of claims 34-42, wherein the amino acid residue in position 7 of said GLP-1 peptide is selected from the group consisting of D-histidine, desamino-histidine, 2-amino-histidine, β-hydroxy-histidine, homohistidine, N^{α}-acetyl-histidine , α-fluoromethyl-histidine, α-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine and 4-pyridylalanine.

44. A compound according to any one of claims 34-43, wherein said GLP-1 peptide is selected from the group consisting of Arg^{34}GLP-1(7-37), $Lys^{38}Arg^{26,34}$GLP-1(7-38), $Lys^{38}Arg^{26,34}$GLP-1(7-38)-OH, $Lys^{38}Arg^{26,34}$GLP-1(7-36), $Aib^{8,22,35}$GLP-1(7-37), $Aib^{8,35}$GLP-1(7-37), $Aib^{8,22}$GLP-1(7-37), $Aib^{8,22,35}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,22}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,22,35}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Arg^{26,34}Lys^{38}$GLP-1(7-38), $Aib^{8,22,35}Arg^{26}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Arg^{26}Lys^{38}$GLP-1(7-38), $Aib^{8,22}Arg^{26}Lys^{38}$GLP-1(7-38), $Aib^{8,22,35}Arg^{34}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Arg^{34}Lys^{38}$GLP-1(7-38), $Aib^{8,22}Arg^{34}Lys^{38}$GLP-1(7-38), $Aib^{8,22,35}Ala^{37}Lys^{38}$GLP-1(7-38), $Aib^{8,35}Ala^{37}Lys^{38}$GLP-1(7-38), $Aib^{8,22}Ala^{37}Lys^{38}$GLP-1(7-38), $Aib^{8,22,35}Lys^{37}$GLP-1(7-37), $Aib^{8,35}Lys^{37}$GLP-1(7-37) and $Aib^{8,22}Lys^{37}$GLP-1(7-38).

45. A compound according to any one of claims 34-44, wherein said GLP-1 peptide is attached to said hydrophilic spacer via the amino acid residue in position 23, 26, 34, 36 or 38 relative to the amino acid sequence SEQ ID No:1.

46. A compound according to any one of claims 34-37, wherein said GLP-1 peptide is exendin-4.

47. A compound according to any one of claims 34-37, wherein said GLP-1 peptide is ZP-10, i.e. HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPSKKKKKK-amide.

48. A compound according to any one of claims 34-47, wherein one albumin binding residue via said hydrophilic spacer is attached to the C-terminal amino acid residue of said GLP-1 peptide.

49. A compound according to claim 48, wherein a second albumin binding residue is attached to an amino acid residue which is not the C-terminal amino acid residue.

50. A compound according to any one of the previous claims, wherein said compound is selected from the group consisting of

$N^{\epsilon 37}$-(2-(2-(2-(dodecylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(2-(2-(2-(17-sulphohexadecanoylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,Lys37]GLP-1(7-37) amide,

$N^{\epsilon 37}$-{2-[2-(2-(15-carboxyhexadecanoylamino)ethoxy)ethoxy]acetyl}-[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(2-(2-(2-(17-carboxyheptadecanoylamino)ethoxy)ethoxy)acetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(2-(2-(2-(19-carboxynonadecanoylamino)ethoxy)ethoxy)acetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,

$N^{\epsilon 38}$-(4-(hexadecanoylamino)-4(S)-carboxybutyryl)-[$Aib^{8,22,35}$,$Arg^{26,34}$,Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$-(2-(2-(2-(hexadecanoylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,$Arg^{26,34}$,Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 37}$(2-[2-(2,6-(S)-Bis-{2-[2-(2-(dodecanoylamino)ethoxy)ethoxy]acetylamino}hexanoylamino)ethoxy]ethoxy}) acetyl-[$Aib^{8,22,35}$]GLP-1(7-37) amide,

$N^{\epsilon 37}$-(2-[2-(2,6-(S)-Bis-{2-[2-(2-(tetradecanoylamino)ethoxy)ethoxy]acetylamino}hexanoylamino)ethoxy]ethoxy}) acetyl-[$Aib^{8,22,35}$]GLP-1(7-37) amide,

$N^{\epsilon 38}$-(2-(2-(2-(4-(hexadecanoylamino)-4(S)-carboxybutyrylamino)ethoxy)ethoxy)acetyl)-[$Aib^{8,22,35}$,$Arg^{26,34}$,Lys^{38}]GLP-1(7-37),

$N^{\epsilon 38}$-(2-{2-[4-[4-(4-Amino-9,10-dioxo-3-sulfo-9,10-dihydro-anthracen-1-ylamino)-2-sulfo-phenylamino]-6-(2-sulfo-phenylamino)-[1,3,5]triazin-2-ylamino]-ethoxy}-ethoxy)-acetyl)-[$Aib^{8,22,35}$,Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$-({2-[2-(2-{2-[2-(2-{2-[2-(15-carboxypentadecanoylamino)-ethoxy]ethoxy}acetylamino)ethoxy]ethoxy}acetyl amino)ethoxy]ethoxy}acetyl)- [$Aib^{8,22,35}$, Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$-([2-(2-{3-[2,5-dioxo-3-(15-carboxypentadecylsulfanyl)-pyrrolidin-1-yl]-propionylamino}ethoxy)ethoxy)acetyl]-[D-Ala^{8},$Lys^{37,38}$]GLP-1-[7-37] amide,

$N^{\epsilon 38}$-(2-(2-(2-(11-(oxalylamino)undecanoylamino)ethoxy)ethoxy)acetyl-)) [$Aib^{8,22,35}$, Ala^{37},Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$({2-[2-(2-{2-[2-(2-(15-carboxy-pentadecanoylamino)-ethoxy]ethoxy}acetylamino)ethoxy]ethoxy}acetyl)[$Aib^{8,22,35}$,Ala^{37},Lys^{38}]GLP-1(7-37) amide,

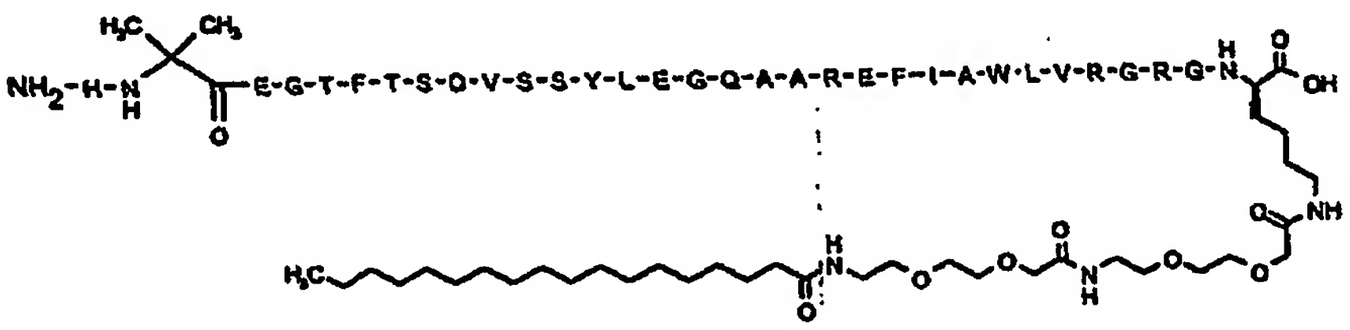
$N^{\epsilon 38}$-((2-{2-[11-(5-Dimethylaminonaphthalene-1-sulfonylamino)undecanoylamino]ethoxy}ethoxy)acetyl [$Aib^{8,22,35}$,Ala^{37},Lys^{38}]GLP-1(7-37) amide,

$N^{\epsilon 38}$([2-(2-{2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetylamino}ethoxy)ethoxy]acetyl) [$Aib^{8,22,35}$,Ala^{37},Lys^{38}]GLP-1(7-37) amide,

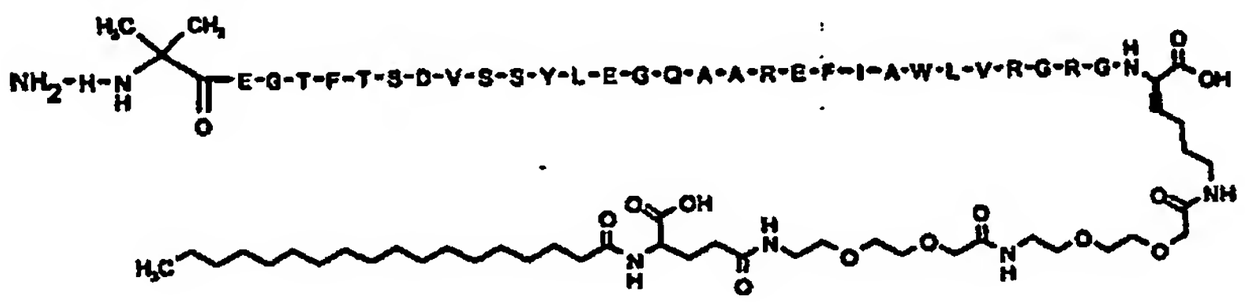
$N^{\epsilon 38}$-2-(2-(2-(octadecanoylamino)ethoxy)ethoxy)acetyl [Aib^{8},$Arg^{26,34}$,$Glu^{22,23,30}$,Lys^{38}]GLP-1 (7-37) amide,

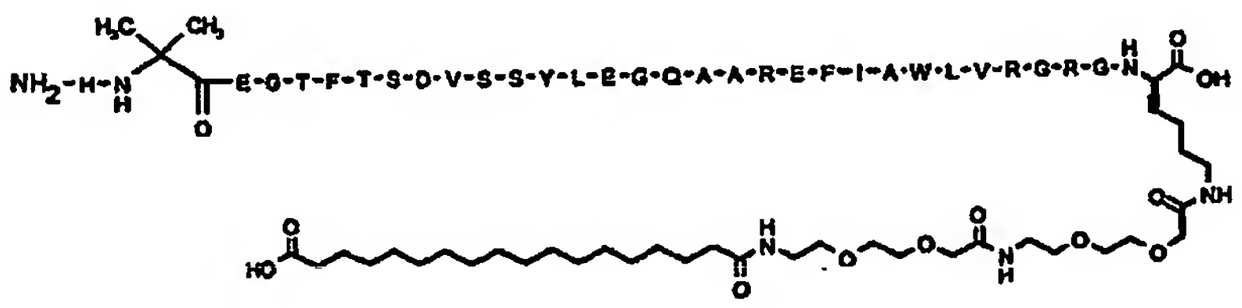
$N^{\epsilon 38}$-2-(2-(2-(eicosanoylamino)ethoxy)ethoxy)acetyl [Aib^{8},$Arg^{26,34}$,$Glu^{22,23,30}$,Lys^{38}]GLP-1(7-37) amide,

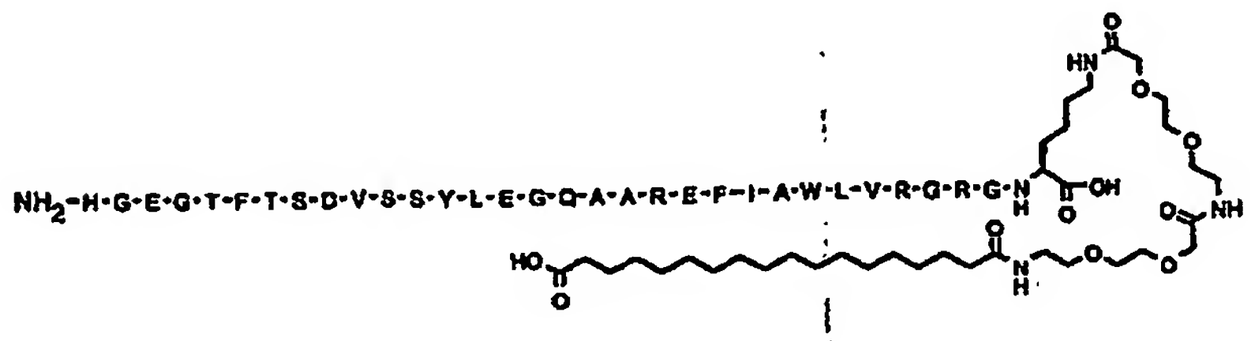
$N^{\epsilon 38}$({2-[2-(2-{2-[2-(4-Carboxy-4-octadecanoylamino-butyrylamino)ethoxy]ethoxy}acetylamino)ethoxy]ehoxy}-acetic acid)[Gly^{8},$Arg^{26,34,36}$,Lys^{38}]GLP-1(7-37),



$N^{\epsilon 38}$-(2-(2-(2-(gamma-glutamyl(N-alfa-hexadecanoyl))aminoethoxy)ethoxy)acetyl)[Aib^{8},Lys^{38}]GLP-1(7-37),







$N^{\epsilon 38}$-(2-(2-(2-(2-(2-(2-(gamma-glutamyl(N-alfa-hexadecanoyl))aminoethoxy)ethoxy)acetyl) aminoethoxy)ethoxy)acetyl)[Aib^{8},Lys^{38}]GLP-1(7-38),
$N^{\epsilon 37}$-(C12oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,
$N^{\epsilon 37}$-(C14oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,
$N^{\epsilon 37}$-(C16oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide,
$N^{\epsilon 37}$-(C18oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide and
$N^{\epsilon 37}$-(C20oyl-aminoethoxy-ethoxyacetyl)[$Aib^{8,22,35}$,Lys^{37}]GLP-1(7-37) amide.

51. A compound according to any one of claims 1-33, wherein said therapeutic polypeptide is a GLP-2 peptide.

52. A compound according to claim 51, wherein said GLP-2 peptide is a DPPIV-protected GLP-2 peptide.

53. A compound according to claim 51, wherein said GLP-2 peptide is Gly^{2}-GLP-2(1-33).

54. A compound according to claim 51, wherein said GLP-2 peptide is $Lys^{17}Arg^{30}$-GLP-2(1-33).

55. A compound according to any one of claims 1-33, wherein said therapeutic polypeptide is human insulin or an analogue thereof.

56. A compound according to claim 55, wherein said therapeutic polypeptide is selected from the group consisting of Asp^{B28}-human insulin, Lys^{B28},Pro^{B29}-human insulin, Lys^{B3},Glu^{B29}-human insulin, Gly^{A21},Arg^{B31},Arg^{B32}-human insulin and des(B30) human insulin.

57. A compound according to any one of claims 1-33, wherein said therapeutic polypeptide is human growth hormone or an analogue thereof.

58. A compound according to any one of claims 1-33, wherein said therapeutic polypeptide is parathyroid hormone or an analogue thereof.

59. A compound according to any one of claims 1-33, wherein said therapeutic polypeptide is human follicle stimulating hormone or an analogue thereof.

60. A compound according to any one of claims 1-33, wherein said therapeutic polypeptide has a molar weight of less than 100 kDa, less than 50 kDa, or less than 10 kDa.

61. A compound according to any one of claims 1-33, wherein said therapeutic polypeptide is selected from the group consisting of a growth factor such as platelet-derived growth factor (PDGF), transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), a somatomedin such as insulin growth factor i (IGF-i), insulin growth factor ii (IFG-ii), erythropoietin (EPO), thrombopoietin (TPO) or angiopoietin, interferon, pro-urokinase, urokinase, tissue plasminogen activator (t-PA), plasminogen activator inhibitor 1, plasminogen activator inhibitor 2, von Willebrandt factor, a cytokine, e.g. an interleukin such as interleukin (IL) 1, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-9, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-20 or IL-21, a colony stimulating factor (CFS) such as GM-CSF, stem cell factor, a tumor necrosis factor such as TNF-α, lymphotoxin-α, lymphotoxin-β, CD40L, or CD30L, a protease inhibitor e.g. aprotinin, an enzyme such as superoxide dismutase, asparaginase, arginase, arginine deaminase, adenosine deaminase, ribonuclease, catalase, uricase, bilirubin oxidase, trypsin, papain, alkaline phosphatase, β-glucoronidase, purine nucleoside phosphorylase or batroxobin, an opioid, e.g. endorphins, enkephalins or non-natural opioids, a hormone or neuropeptide, e.g. calcitonin, glucagon, gastrins, adrenocorticotropic hormone (ACTH), cholecystokinins, lutenizing hormone, gonadotropin-releassing hormone, chorionic gonadotropin, corticotrophin-releasing factor, vasopressin, oxytocin, antidiuretic hormones, thyroid-stimulating hormone, thyrotropin-releasing hormone, relaxin, prolactin, peptide YY, neuropeptide Y, pancreastic polypeptide, leptin, CART (cocaine and amphetamine regulated transcript), a CART related peptide, perilipin, melanocortins (melanocyte-stimulating hormones) such as MC-4, melanin-concentrating hormones, natriuretic peptides, adrenomedullin, endothelin, secretin, amylin, vasoactive intestinal peptide (VIP), pituary adenylate cyclase activating polypeptide (PACAP), bombesin, bombesin-like peptides, thymosin, heparin-binding protein, soluble CD4, hypothalmic releasing factor, melanotonins and analogues thereof.

62. A pharmaceutical composition comprising a compound according to any one of claims 1-61, and a pharmaceutically acceptable excipient.

63. The pharmaceutical composition according to claim 62, which is suited for parenteral administration.

64. Use of a compound according to any one of the claims 1-61 for the preparation of a medicament.

65. Use of a compound according to any one of the claims 34-50 for the preparation of a medicament for the treatment or prevention of hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, obesity, hypertension, syndrome X, dyslipidemia, cognitive disorders, atheroschlerosis, myocardial infarction, coronary heart disease and other cardiovascular disorders, stroke, inflammatory bowel syndrome, dyspepsia and gastric ulcers.

66. Use of a compound according to any one of the claims 34-50 for the preparation of a medicament for delaying or preventing disease progression in type 2 diabetes.

67. Use of a compound according to any one of the claims 34-50 for the preparation of a medicament for decreasing food intake, decreasing β-cell apoptosis, increasing β-cell funtion and β-cell mass, and/or for restoring glucose sensitivity to β-cells.

68. Use of a compound according to any one of claims 51-54 for the preparation of a medicament for the treatment of small bowel syndrome, inflammatory bowel syndrome or Crohns disease.

69. Use of a compound according to any one of claims 55-56 for the preparation of a medicament for the treatment or prevention of hyperglycemia, type 1 diabetes, type 2 diabetes or β-cell deficiency.

ABSTRACT

Novel polypeptide derivatives having protracted profile of action.

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<120> Novel GLP-1 derivatives

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<140> 6692.010-DK

<141> 2003-11-25

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<170> PatentIn version 3.1

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Gln	Ala	Ala	Lys	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Arg	Gly
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<223> Xaa at position 2 is Ala, Gly, Val, Leu, Ile, Lys, Aib, (1-aminocyclopropyl) carboxylic acid, (1-aminocyclobutyl) carboxylic acid,(1-aminocyclopentyl) carboxylic acid, (1-aminocyclohexyl) carboxylic acid, (1-aminocycloheptyl) carboxylic acid or (1-aminocyclooctyl) carboxylic acid.

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<223> Xaa at position 17 is Gln, Glu, Lys or Arg.

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<223> Xaa at position 20 is Lys, Glu or Arg.

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<223> Xaa at position 27 is Val or Lys.

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<223> Xaa at position 28 is Lys, Glu, Asn or Arg.

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<223> Xaa at position 39 is Ser, amide or is absent.

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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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<222> (2)..(2)

<223> Xaa at position 2 is Ala, Gly, Val, Leu, Ile, Lys, Aib, (1-aminocyclopropyl) carboxylic acid, (1-aminocyclobutyl) carboxylic acid, (1-aminocyclopentyl) carboxylic acid, (1-aminocyclohexyl) carboxylic acid, (1-aminocycloheptyl) carboxylic acid or (1-aminocyclooctyl) carboxylic acid.

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<223> Xaa at position 1 is L-histidine, D-histidine, desamino-histidine, 2-amino-histidine, beta-hydroxy-histidine, homohistidine, N-alpha-acetyl-histidine, alpha-fluoromethyl-histidine, alpha-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine, or 4-pyridylalanine.

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<223> Xaa at position 32 is Lys, amide or is absent.

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Xaa Ala Ala Xaa Glu Phe Ile Xaa Trp Leu Val Xaa Xaa Xaa Xaa Xaa
20 25 30

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<223> Amidation of carboxy group

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Ser Gly Ala Pro Pro Pro Ser
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<223> Amidation of carboxyl group

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Ser Gly Ala Pro Pro Ser Lys Lys Lys Lys Lys Lys